IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: OKAMURA=2E

In re Application of:	)	Group Art Unit: 1646
OKAMURA et al.	)	Examiner: D. Jiang
Appln. No.: 09/373,230	)	Washington, D.C.
Date Filed: August 12, 1999	)	November 14, 2005
For: IFN-γ PRODUCTION INDUCING PROTEIN AND MONOCLONAL ANTIBODY OF THE SAME	)	

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Haruki OKAMURA, declare and state as follows:

1. I am a citizen of Japan residing at Nakahozumi, 2-12-32, Ibaraki-shi, Osaka, Japan.

2. I have graduated from Osaka University, Faculty of Science (Biology) and received a doctorate of Science at Osaka University in 1976.

3. I have been working for Hyogo College of Medicine since 1976. I am the professor, Laboratory of Host Defenses, Institute for Advanced Medical Science, Hyogo College of Medicine.

4. I have been engaged in research in the field of microorganisms and cytokines, particularly, interleukin-18 (IL-18). A true copy of my curriculum vitae is attached hereto.

5. I am one of the inventors on the above-identified patent application and am thoroughly familiar with the specification.

6. I and other co-inventors of the present invention screened a protein which induces interferon-γ (IFN-γ) production by

immunocompetent cells. As a result, I and other co-inventors first discovered a novel protein, i.e., interleukin 18 (IL-18), from mouse and then revealed its amino acid sequence and nucleotide sequence; an amino acid sequence of SEQ ID NO:3 and a nucleotide sequence of SEQ ID NO:4 in the original specification of the above-identified patent application (called "the specification", hereinafter), respectively.

7. At the time the invention was made, once the above information were available, then the skilled person in the art could have easily understood and engineered various variants of the protein with the amino acid sequence of SEQ ID NO:3, i.e., a variety of polypeptides which are homologous to the protein, where one or more amino acids in SEQ ID NO:3 are replaced with other amino acids, one or more amino acids are added to the amino acid sequence of SEQ ID NO:3, particularly, to the N- or C-terminal region in SEQ ID NO:3; and/or one or more amino acids are deleted from the amino acid sequence of SEQ ID NO:3, particularly, from the N- or C-terminal region of SEQ ID NO:3, without altering the inherent biological properties of the protein with the amino acid sequence of SEQ ID NO:3.

8. Based on these, I and other co-inventors disclosed such variants at page 9, 3rd paragraph and page 15, 3rd paragraph through page 16, 1st paragraph in the specification.

9. As regards such variants, they should not be limited to specific ones as long as they are the sequence variants of the protein with the amino acid sequence of SEQ ID NO:3 and have substantially the same biological properties as the protein, especially, an activity of inducing interferon-γ production by immuno-competent cells and activating the cytotoxicity of killer cells, where the activation is augmented by interleukin 2.

10. The specification is silent about the specific homology of the above-identified variants to the protein with the amino acid sequence of SEQ ID NO:3. However, I and other co-inventors had in mind that such variants are those which are at least 90% homologous to but different from the protein with the amino acid sequence of SEQ ID NO:3, based on the previous publications which show the percentages of homology among interleukins (ILs) of animals, particularly, rodents such as mice and rats. Examples of such publications are listed in "16. References", i.e., references (a) to (f).

11. In the specification, I and other co-inventors disclosed no substantial concrete example of such variants but they were surely within our envisaged scope, because I and other co-inventors had recognized at the time the invention was made a person skilled in the art could have easily understood and engineered such variants based on the disclosure of the specification, as well as the above-identified publications and conventional recombinant DNA technology as shown, for example, in "*Recombinant DNA*", Second Edition, Chapters 11 and 23, edited by James D. Watson *et al.*, published by W. H. Freeman and Company, 1992.

12. Besides, for example, US Patent Nos. 4,798,885 and 5,494,792, which were filed at the USPTO before the filing date of the present application and which claim for proteins, polypeptides, and their sequence variants with the desired biological activity, for example, at least 90% or 95% homologous to the specific amino acid sequence of any one of such proteins and polypeptides, were patented without substantial concrete disclosure of amino acid sequences of such variants in the above-identified specifications.

13. This means that, even at the time or before 1993 at which the above US Patents were made, the person skilled in the art to which it pertains could have obtained variants of any proteins and polypeptides, if only the amino acid sequence of such proteins and polypeptides were once revealed.

14. Under these conditions, I believe that the specification has been sufficiently disclosed in such a manner that it does surely enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make the invention commensurate in scope with the claims for variants which are at least 90% homologous to but different from the protein with the amino acid sequence of SEQ ID NO:3.

15. I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

16. References:

(a) *Nucleic Acids Research*, D. R. Cohen, A. J. Hapel et al., Vol. 14, No. 9, pp. 3641-3658, 1986;

(b) *J. Biochem.*, Tsutomu Nishida et al., Vol. 105, pp. 351-357, 1989;

(c) *Cytokine*, Klaus Uberla et al., Vol. 3, No. 1, pp. 72-81, 1991;

(d) *The Journal of Biological Chemistry*, Wolfgang Northemann et al., Vol. 264, No. 27, pp. 16072-16082, 1989;

(e) *Biochemical and Biophysical Research Communications*, Fadi G. Lakkis et al., Vol. 197, No. 2, pp. 612-618, 1993;

(f) *Biochemical and Biophysical Research Communications*, Richard E. Goodman et al., Vol. 189, No. 1, pp. 1-7, 1992;

(g) "*Recombinant DNA*", Second Edition, Chapters 11 and 23, James D. Watson et al., published by W. H. Freeman and Company, 1992;

(h) US Patent No. 4,798,885; and

(i) US Patent No. 5,494,792.

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DATE: 14th day of November, 2005

CURRICULUM VITAE

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EDUCATION:

1970	Graduated from Osaka University, Faculty of Science (Biology)
1976	Degree of Ph.D, Osaka University
1976	Scholarship Student, Institute for Microbial Diseases, Department of Measles, Osaka University

BRIEF CHRONOLOGY OF EMPLOYMENT:

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REFERENCES:

(1) Okamura, H., Tsutsui, H., Komatsu,T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y., Hattori, K., Akita, K., Namba, M., Tanabe, F., Konishi, K., Fukuda, S., and Kurimoto, M., Cloning of a new cytokine that induces IFN-γ production by T cells. Nature 378, 88-91, 1995

(2) Ushio, S., Namba, M., Okura, T., Hattori, K., Nukata, Y., Akita, K., Tanabe, F., Konishi, K., Micaleff, M., Fijii, M., Torigoe, K., Tanimoto, T., Fukuda, S., Ikeda, M., Okamura, H., and Kurimoto, M., Cloning of the cDNA for Human IFN-γ-Inducing Factor, Expression in Escherichia coli, and Studies on the Biological Activities of the Protein lipid A. J. Immunol., 156, 4274-4, 1996

(3) Tsutsui, H., Nakanishi, K., Matsui, K., Higashino, K., Okamura, H., Miyazawa, Y., and Kaneda, K., IFN-γ Inducing Factor Up-Regulates Fas Ligand-Mediated Cytotoxic Activity of Murine Natural Killer Cell Clones. J. Immunol., 157, 3367-3973, 1996

(4) Tao, D., Ohashi, K., Tayano, T., Kurimoto, M., and Okamura, H., Interferon-γ-Inducing Factor, a Novel Cytokine, Enhances Fas Ligand-Mediated Cytotoxicity of Murine T Helper 1 Cells. Cell. Immunol., 173, 230- 235, 1996

(5) Ahn, H., Maruo, S., Tomura, M., Mu, J., Hamaoka, T., Nakanishi, K., Clark, S., Kurimoto,M., Okamura, H., and Fujiwara,H., A mechanism underlying synergy between IL-12 and IFN-γ-inducing factor (IL-18) in enhanced production of IFN-γ. J. Immunol., 159, 2125-2131, 1997

(6) Zhang, T., Kawakami, K., Qureshi, M.H., Okamura, H., Kurimoto, M., and Satoh, A., Interleukin-12(IL-12) and IL-18 synergistically induce the fungicidal activity of murine peritoneal exudate cells against Cryptococcus neoformans through production of gamma interferon by natural killer cells. Infect. Immun., 65, 3594-3599, 1997

(7) Stoll,S., Muller, G., Kurimoto, M., Saloga, J., Tanimoto, T., Yamauchi, H., Okamura, H., Knop, J., and Enk, AH., Production of IL-18 (IFN-γ-inducing factor) mRNA and functional protein by murine keratinocytes. J. Immunol., 159, 298-302, 1997

(8) Gu, Y., Kuida, K., Tsutsui, H., Ku, G., Fleming, M, A., Hayashi, N., Higashino, K., Okamura, H., Nakanishi, K., Kurimoto, M., Tanimoto, T., Flavell, R, A., Sato, V., Harding, M, W., Livingston DJ., and Su MS, Activation of Interferon-γ inducing factor mediated by Interferon-1 converting enzyme. Science, 275, 206-209, 1997

(9) Yoshimoto, T., Okamura, H., Tagawa, Y., Iwakura, Y., and Nakanishi, K., Interleukin-18 (IL-18) together with IL-12 inhibits IgE production by induction of IFN-γ production from activated B cells. Proc. Natl. Acad. Sci. USA., 94, 3948-3953, 1997

(10) Tsutsui, H., Matsui, K., Kawada, N., Hyodo, Y., Hayashi, N., Okamura, H., Higashino, K., and Nakanishi, K., IL-18 accounts for both TNF-α-and Fas Ligand-mediated hepatotoxic pathways in endotoxin-induced liver injury in mice. J. Immunol., 159, 3961-3967, 1997

(11) Udagawa, N., Horwood, NJ., Elliott, J., Mackay, A., Owen, J., Okamura, H., Kurimoto,M., Chambers, TJ., Martin, TJ., and Gillespie, Mt., Interleukin-18 (interferon-γ-inducing factor) is produced by osteoclasts and acts granulocyte/macrophage colony-stimulating factor and not via interferon-γ to inhibit osteoclast formation. J. Exp. Med., 185, 1005-1012, 1997

(12) Yoshimoto, T., Takeda, K., Tanaka, T., Ohkusu, K., Kashiwamura, S-I., Okamura,H., Akira, S., and Nakanishi, K., IL-12 up-regulates IL-18R expression on T cells and B cells : synergism with IL-18 for IFN-γ production. J. Immunol., 161, 3400-3407, 1998

(13) Takeda, K., Tsutsui, T., Yoshimoto,T., Adachi, O., Yoshida, N., Kishimoto, T., Okamura, H., Nakanishi, K., and Akira, S., Defective NK cell activity and Th1 response in IL-18-deficient mice. Immunity, 8, 383-390, 1998

(14) Osaki, T., Peron, J. M., Cai, Q., Okamura, H., Robbins, P. D., Kurimoto, M., Lotze, M. T., and Tahara, H., IFN-gamma-inducing factor/IL-18 administration mediate IFN-gamma and Il-12-independent antitumor effects. J.Immunol., 160, 1742-1749, 1998

(15) Okamura, H., Tsutsui, T., Kashiwamura, S-I., Yoshimoto,T., and Nakanishi, K., Interleukin-18 (IL 18) : a novel cytokine that augments both innate and augments both innate and acquired immunity. Adv. Immunol., 70, 281-312, 1998

(16) Okamura, H., Kashiwamura, S-I., Tsutsui, H., Yoshimoto, T., and Nakanishi, K., Regulation of interferon-gamma (IFN-γ) production by IL-12 and IL-18. Curr. Opin. Immunol., 10, 259-264, 1998

(17) Osaki, T., Hashimoto, W., Gambotto, A., Okamura, H., Robbins, P. D., Kurimoto, M., Lotz, M. T., Tahara, H., Potent anti-tumor effects mediated by local expression of the mature form of interferon-gamma inducing factor, interleukin-18 (IL-18). Gene Therapy, 6, 808-815, 1999.

(18) Hyodo, Y., Matsui, K., Hayashi, N., Tsutsui, H., Kashiwamura, S., Yamauchi, H., Hiroishi, K., Takeda, K., Tagawa, Y. Iwakura, Y., Kayagaki, N., Kurimoto, M., Okamura, H., Hada, T., Yagita, H., Akira, S., Nakanishi, K., Higashino, K., IL-18 up-regulates perforine-mediated NK activity without increasing perforine messenger RNA expression by binding to constitutively expressed IL-18 receptor. J. Immunol., 162, 1662-1668, 1999

(19) Rothe, H., Hausmann, A., Casteels, K.,Okamura, H., Kurimoto, M., Burkart, V., Mathieu, C., and Kolb, H., IL-18inhibits diabetes development in nonobese diabetic mice by couterregulation of Th1-dependent destructive insulitis. J. Immunol., 163, 1230-1236, 1999

(20) Hashimoto, W., Osaki, T., Okamura, H., Robbins, P.D., Kurimoto, M., Nagata, S., Lotze, M.T., and Tahara, H. Differential antitumor effects of administration of recombinant IL-18 or recombinant IL-12 are mediated primarily by Fas-Fas ligand- and perforin-induced tumor apoptosis, respectively. J. Immunol., 163, 583-9, 1999

(21) Yogi, Y., Endoh, M., Tanaka, T., Akira, S., Okamura, H., and Nomaguchi, H. Bacteria killing by macrophages via NF-IL6 gene dependent mechanism: the susceptibility to Mycobacterium leprae in NF-IL6 knockout mice. Nihon Hansenbyo Gakkai Zasshi., 68, 97-108, 1999

(22) Yamamoto, T., Moriwaki, Y., Matsui, K., Takahashi, S., Tsutsui, H., Yoshimoto, T., Okamura, H., Nakanishi, K., Kurosawa, Y., Yamaguchi, S., and Sasaki, Y., Higashino K. High IL-18 (interferon-gamma inducing factor) concentration in a purine nucleoside phosphorylase deficient patient. Arch Dis Child., 81, 179-80, 1999

(23) Tsutsui, H., Kayagaki, N., Kuida, K., Nakano, H., Hayashi, N., Takeda, K., Matsui, K., Kashiwamura, S., Hada, T., Akira, S., Yagita, H., Okamura, H., and Nakanishi, K., Caspase-1-independent, Fas/Fas ligand-mediated IL-18 secretion from macrophages causes acute liver injury in mice. Immunity, 11, 359-67, 1999

(24) Yoshimoto, T., Tsutsui, H., Tominaga, K., Hoshino, K.,Okamura, H., Akira, S., Paul, W.E., and Nakanishi, K., IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. Proc.Natl.Acad.Sci.USA., 96, 13962-6, 1999

(25) Nakata, A., Tsujimura, T., Sugihara, A.,Okamura, H., Iwasaki, T., Shinkai, K., Iwata, N., Kakishita, E., Akedo, H., and Terada, N., Inhibition by interleukin 18 of osteolytic bone metastasis by human breast cancer cells. Anticancer, Res., 19(5B), 4131-8, 1999

(26) Kodama, T., Matsuyama, T., Kuribayashi, K., Nishioka, Y., Sugita, M., Akira, S., Nakanishi, K., and Okamura, H., IL-18 deficiency selectively enhances allergen-induced eosinophilia in mice. J Allergy.Clin.Immunol., 105(1 Pt 1), 45-53, 2000

(27) Tominaga. K., Yoshimoto. T., Torigoe. K., Kurimoto. M., Matsui, K., Hada, T., Okamura, H., and Nakanishi, K., IL-12 synergizes with IL-18 or IL-1beta for IFN-gamma production from human T cells. Int, Immunol., 12, 151-60, 2000

(28) Ohkusu, K., Yoshimoto, T., Takeda, K., Ogura, T., Kashiwamura, Si., Iwakura, Y., Akira, S., Okamura, H., and Nakanishi, K., Potentiality of interleukin-18 as a useful reagent for treatment and prevention of leishmania major infection. Infect. Immun., 68, 2449-56, 2000

(29) Ida, A., Tsuji, Y., Muranaka, J., Kanazawa, R., Nakata, Y., Adachi, S., Okamura, H., and Koyama, K., IL-18 in pregnancy; the elevation of IL-18 in maternal peripheral blood during labour and complicated pregnancies. J. Reprod. Immunol., 47, 65-74, 2000

(30) Tsutsui, H., Matsui, K., Okamura, H., and Nakanishi, K., Pathophysiological roles of interleukin-18 in inflammatory liver diseases. Immunol, Rev., 174, 192-209, 2000

(31) Chang, J.T., Segal, B.M., Nakanishi, K., Okamura, H. and Shevach, E.M., The costimularory effect of IL-18 on the induction of antigen-specific IFN-γ production by resting T cells is IL-12 dependent and is mediated by up-regulation of the IL-12 receptor ß2 subunit. Eur.J. Immunol., 30, 1113-1119, 2000

(32) Fujimori, Y., Takatsuka, H., Takemoto, Y., Hara, H., Okamura, H., Nakanishi, K., Kakishita, E. Elevated interleukin (IL)-18 levels during acute graft-versus-host disease after allogeneic bone marrow transplantation. British J. Haematology., 109, 652-657, 2000

(33) Yamanaka, K., Tanaka, M., Tsutsui, H., Kupper, T.S., Asahi, K., Okamura, H., Nakanishi, K., Suzuki, M., Kayagaki, N., Black, R.A., Miller, D.K., Nakashima, K., Shimizu, M. and Mizutani., H. Skin-specific caspase-1 transgenic mice show cutaneous apoptosis and pre-shock condition with a high serum level of IL-18. J. Immunol., 165, 997-1003, 2000

(34) Yoshimoto, T., Mizutani, H., Tsutsui, H., Noben-Trauth, N., Yamanaka, K-I., Tanaka, M., Izumi, S., Okamura, H., Paul, W.E. and Nakanishi, K. IL-18 induction of IgE: dependence on CD4+T cells, IL-4 and STAT6. Nature Immunol., 1, 132-137, 2000

(35) Chikano, S., Sawada, K., Shimoyama, T., Kashiwamura, S-I., Sugihara, A., Sekikawa, K., Terada, N., Nakanishi, K. and Okamura, H. IL-18 and IL-12 induce intestinal inflammation and fatty liver in mice in an IFN-γ dependent manner. Gut, 47, 779-786, 2000

(36) Shigehara, K., Shijubo, N., Ohmichi, M., Yamada, G., Takahashi, R., Okamura, H., Kurimoto, M., Hiraga, Y., Tatsuno, T., Abe, S. and Sato, N. Increased levels of interleukin-18 in patients with pulmonary sarcoidosis. Am. J. Respir. Crit. Care. Med., 162, 1979-1982, 2000

(37) Tanaka, F., Hashimoto, W., Okamura, H., Robbins, P.D., Lotze, M.T. and Tahara, H. Rapid generation of potent and tumor-specific cytotoxic T lymphocytes by interleukin 18 using dendritic cells and natural killer cells. Cancer Res., 60, 4838-4844, 2000

(38) Yamada, G., Shijubo, N., Shigehara, K., Okamura, H., Kurimoto, M and Abe, S. Increased levels of circulating interleukin-18 in patients with advanced tuberculosis. Am. J. Respir. Crit. Care. Med., 161, 1786-1789, 2000

(39) Kuhara, T., Iigo, M., Itoh, T., Ushida, Y., Sekine,K., Terada, N., Okamura, H., and Tsuda, H. Orally administered lactoferrin exerts an antimetastatic effect and enhances production of IL-18 in the intestinal epithelium. Nutr. Cancer, 38, 192-199, 2000

(40) Eberl, M., Beck, E., Coulson, P.S., Okamura, H., Wilson, R.A., and Mountford, A.P. IL-18 potentiates the adjuvant properties of IL-12 in the induction of a strong Th1 type immune response against a recombinant antigen. Vaccine, 18, 2002-2008, 2000

(41) Makiishi-Shimobayashi, C., Tsujimura, T., Iwasaki, T., Yamada, N., Sugihara, A., Okamura, H., Hayashi, S., and Terada, N. Interleukin-18 up-regulates osteoprotegerin expression in stromal/osteoblastic cells. Biochem. Biophys. Res. Commun., 281, 361-366

(42) Seki, E., Tsutsui, H., Nakano, H., Tsuji, N., Hoshino, K., Adachi, O., Adachi, K., Futatsugi, S., Kuida, K., Takeuchi, O., Okamura, H., Fujimoto, J., Akira, S., and Nakanishi, K. Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1beta. J. Immunol., 166, 2651-2657, 2001

(43) Shigehara, K., Shijubo, N., Ohmichi, M., Takahashi, R., Kon. S., Okamura, H., Kurimoto, M., Hiraga, Y., Tatsuno, T., Abe, S., and Sato, N. IL-12 and IL-18 are increased and stimulate IFN-gamma production in sarcoid lungs. J. Immunol., 166, 642-649, 2001

(44) Sugawara, S., Uehara, A., Nochi, T., Yamaguchi, T., Ueda, H., Sugiyama, A., Hanzawa, K., Kumagai, K., Okamura, H., and Takada, H. Neutrophil proteinase 3-mediated induction of bioactive IL-18 secretion by human oral epithelial cells. J. Immunol., 167, 6568-6575, 2001

(45) Tsuji, Y., Tamaoki, T.H., Hasegawa, A., Kashiwamura, S., Iemoto, A., Ueda, H., Muranaka, J., Adachi, S., Furuyama, J., Okamura, H., and Koyama, K. Expression of interleukin-18 and its receptor in mouse ovary. Am. J. Reprod. Immunol., 46, 349-357, 2001

(46) Adachi, K., Tsutsui, H., Kashiwamura , S., Seki , E., Nakano, H., Takeuchi, O., Takeda, K., Okumura, K., Van, Kaer, L., Okamura, H., Akira, S., and Nakanishi, K. Plasmodium berghei infection in mice induces liver injury by an IL-12- and toll-like receptor/myeloid differentiation factor 88-dependent mechanism. J. Immunol., 167, 5928-5934, 2001

(47) Nomaguchi, H., Jahan, N., Mandal, B.C., Yogi, Y., Kawatsu, K., Yoshizawa, Y., Okamura, H., and Makino, M. IL-12 and IL-18 synergistically induce the bactericidal activity of murine peritoneal cells against M. leprae. Nihon, Hansenbyo, Gakkai, Zasshi, 70, 113-119, 2001

(48) Ogura, T., Ueda, H., Hosohara, K., Tsuji, R., Nagata, Y., Kashiwamura, S., and Okamura, H. Interleukin-18 stimulates hematopoietic cytokine and growth factor formation and augments circulating granulocytes in mice. Blood, 98, 2101-2107, 2001

(49) Itoi, H., Fujimori, Y., Tsutsui, H., Matsui, K., Futatsugi, S., Okamura, H., Hara, H., Hada, T., Kakishita, E., and Nakanishi, K. Fas ligand-induced caspase-1-dependent accumulation of interleukin-18 in mice with acute graft-versus-host disease. Blood, 98, 235-237, 2001

(50) Shida, K., Shiratori, I., Matsumoto, M., Fukumori, Y., Matsuhisa, A., Kikkawa, S., Tsuji, S., Okamura, H., Toyoshima, K., and Seya, T. An alternative form of IL-18 in human blood plasma: complex formation with IgM defined by monoclonal antibodies. J. Immunol., 166, 6671-6679, 2001

(51) Nakanishi, K., Yoshimoto, T., Tsutsui, H., and Okamura, H. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. Cytokine, Growth, Factor, Rev., 12, 53-72, 2001

(52) Wang, W., Tanaka, T., Okamura, H., Sugita, M., Higa, S., Kishimoto, T., and Suemura, M. Interleukin-18 enhances the production of interleukin-8 by eosinophils. Eur. J. Immunol., 1010-1016, 2001

(53) Nakanishi, K., Yoshimoto, T., Tsutsui, H., and Okamura, H. Interleukin-18 regulates both Th1 and Th2 responses. Annu. Rev. Immunol ., 423-474, 2001

(54) Makiishi-Shimobayashi, C., Tsujimura, T., Iwasaki, T., Yamada, N.,Sugihara, A., Okamura, H., Hayashi, S., and Terada, N. Interleukin-18 up-regulates osteoprotegerin expression in stromal/osteoblastic cells. Biochem. Biophys. Res. Commun., 281, 361-366, 2001

(55) Seki, E., Tsutsui, H., Nakano, H., Tsuji, N., Hoshino, K., Adachi, O., Adachi, K., Futatsugi, S., Kuida, K., Takeuchi, O., Okamura, H., Fujimoto, J., Akira, S., and Nakanishi, K. Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1beta. J.Immunol., 166, 2651-2657, 2001

(56) Shigehara, K., Shijubo, N., Ohmichi, M., Takahashi, R., Kon, S., Okamura, H., Kurimoto, M., Hiraga, Y., Tatsuno, T., Abe, S., and Sato, N. IL-12 and IL-18 are increased and stimulate IFN-gamma production in sarcoid lungs. J. Immunol., 166, 642-649, 2001

(57) Sugawara, S., Uehara, A., Nochi, T., Yamaguchi, T., Ueda, H., Sugiyama, A., Hanzawa, K., Kumagai, K., Okamura, H. and Takada, H. Neutrophil proteinase 3-mediated induction of bioactive IL-18 secretion by human oral epithelial cells. J. Immunol., 167, 6568-6575, 2001.

(58) Tsuji, Y., Tamaoki, T.H., Hasegawa, A., Kashiwamura, S., Iemoto, A. Ueda, H., Muranaka, J., Adachi, S., Furuyama, J., Okamura, H. and Koyama, K. Expression of interleukin-18 and its receptor in mouse ovary. Am. J. Reprod. Immunol., 46, 349-357, 2001.

(59) Adachi, K., Tsutsui, H., Kashiwamura, S., Seki, E., Nakano, H., Takeuchi, O., Takeda, K., Okumura, K., Van, Kaer, L., Okamura, H., Akira, S. and Nakanishi, K. Plasmodium berghei infection in mice induces liver injury by an IL-12- and toll-like receptor/myeloid differentiation factor 88-dependent mechanism. J. Immunol., 167, 5928-5934, 2001.

(60) Nomaguchi, H., Jahan, N., Mandal, B.C., Yogi, Y., Kawatsu, K., Yoshizawa, Y., Okamura, H. and Makino, M. IL-12 and IL-18 synergistically induce the bactericidal activity of murine peritoneal cells against M. leprae. Nihon Hansenbyo Gakkai Zasshi, 70, 113-119, 2001.

(61) Ogura, T., Ueda, H., Hosohara, K., Tsuji, R., Nagata, Y., Kashiwamura, S. and Okamura H. Interleukin-18 stimulates hematopoietic cytokine and growth factor formation and augments circulating granulocytes in mice. Blood, 98, 2101-2107, 2001.

(62) Mori, I., Hossain, M.J., Takeda, K., Okamura, H., Imai, Y., Kohsaka, S., and Kimura, Y. Impaired microglial activation in the brain of IL-18-gene-disrupted mice after neurovirulent influenza A virus infection. Virology, 287, 163-170, 2001.

(63) Itoi, H., Fujimori, Y., Tsutsui, H., Matsui, K., Futatsugi, S., Okamura, H., Hara, H., Hada, T., Kakishita, E. and Nakanishi, K. Fas ligand-induced caspase-1-dependent accumulation of interleukin-18 in mice with acute graft-versus-host disease. Blood, 98, 235-237, 2001.

(64) Shida, K., Shiratori, I., Matsumoto, M., Fukumori, Y., Matsuhisa, A., Kikkawa, S., Tsuji, S., Okamura, H., Toyoshima, K. and Seya, T. An alternative form of IL-18 in human blood plasma: complex formation with IgM defined by monoclonal antibodies. J. Immunol., 166, 6671-6679, 2001.

(65) Wang, W., Tanaka, T., Okamura, H., Sugita, M., Higa, S., Kishimoto, T. and Suemura, M. Interleukin-18 enhances the production of interleukin-8 by eosinophils. Eur. J. Immunol., 31, 1010-1016, 2001.

(66) Makiishi-Shimobayashi, C., Tsujimura, T., Iwasaki, T., Yamada, N., Sugihara, A., Okamura, H., Hayashi, S. and Terada, N. Interleukin-18 up-regulates osteoprotegerin expression in stromal/osteoblastic cells. Biochem. Biophys. Res. Commun., 281, 361-366, 2001.

(67) Seki, E., Tsutsui, H., Nakano, H., Tsuji, N., Hoshino, K., Adachi, O., Adachi, K., Futatsugi, S., Kuida, K., Takeuchi, O., Okamura, H., Fujimoto, J., Akira, S. and Nakanishi, K. Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1beta. J. Immunol., 166, 2651-2657, 2001.

(68) Shigehara, K., Shijubo, N., Ohmichi, M., Takahashi, R., Kon, S., Okamura, H., Kurimoto, M., Hiraga, Y., Tatsuno, T., Abe, S. and Sato, N. IL-12 and IL-18 are increased and stimulate IFN-gamma production in sarcoid lungs. J. Immunol., 166, 642-649, 2001.

(69) Minagawa, K., Tsuji, Y., Ueda, H., Koyama, K., Tanizawa, K., Okamura, H. and Hashimoto-Tamaoki, T. Possible correlation between high levels of IL-18 in the cord blood of pre-term infants and neonatal development of periventricular leukomalacia and cerebral palsy. Cytokine, 17, 164-170, 2002.

(70) Singh, R.P., Kashiwamura, S., Rao, P., Okamura, H., Mukherjee, A. and Chauhan, V.S. The role of IL-18 in blood-stage immunity against murine malaria Plasmodium yoelii 265 and Plasmodium berghei ANKA. J. Immunol., 168, 4674-4681, 2002.

(71) Kokai, M., Kashiwamura, S., Okamura, H., Ohara, K. and Morita, Y. Plasma interleukin-18 levels in patients with psychiatric disorders. J. Immunother., 25(Suppl.1), S68-71, 2002.

(72) Futani, H., Okayama, A., Matsui, K., Kashiwamura, S., Sasaki, T., Hada, T., Nakanishi, K., Tateishi, H., Maruo, S. and Okamura, H. Relation between interleukin-18 and PGE2 in synovial fluid of osteoarthritis: a potential therapeutic target of cartilage degradation. J. Immunother., 25, S61-64, 2002.

(73) Iwasaki, T., Yamashita, K., Tsujimura, T., Kashiwamura, S., Tsutsui, H., Kaisho, T., Sugihara, A., Yamada, N., Mukai, M., Yoneda, T., Okamura, H., Akedo, H. and Terada, N. Interleukin-18 inhibits osteolytic bone metastasis by human lung cancer cells possibly through suppression of osteoclastic bone-resorption in nude mice. J. Immunother., 25(Suppl.1), S52-60, 2002.

(74) Kimura-Shimmyo, A., Kashiwamura, S., Ueda, H., Ikeda, T., Kanno, S., Akira, S., Nakanishi, K., Mimura, O. and Okamura, H. Cytokine-induced injury of the lacrimal and salivary glands. J. Immunother., 25(Suppl.1), S42-51, 2002.

(75) Kashiwamura, S., Ueda, H. and Okamura, H. Roles of interleukin-18 in tissue destruction and compensatory reactions. J. Immunother., 25(Suppl.1), S4-S11, 2002.

(76) Lotze, M.T., Tahara, H. and Okamura, H. Interleukin-18 as a novel, distinct, and distant member of the interleukin-1 family promoting development of the adaptive immune response: the interleukin-18 issue of the Journal of Immunotherapy. J. Immunother, 25(Suppl.1), S1-S3, 2002.

(77) Kuribayashi, K., Kodama, T., Okamura, H., Sugita, M., and Matsuyama, T. Effects of post-inhalation treatment with interleukin-12 on airway hyper-reactivity, eosinophilia and interleukin-18 receptor expression in a mouse model of asthma. Clin. Exp. Allergy, 32, 641-649, 2002.

(78) Uchimura, T., Motomiya, Y., Okamura, H., Hashiguchi, T., Miura, M., Uji, Y., Iwamoto, H. and Maruyama, I. Marked increases in macrophage colony-stimulating factor and interleukin-18 in maintenance hemodialysis patients: comparative study of advanced glycation end products, carboxymethyllysine and pentosidine. Nephron, 90, 401-407, 2002.

(79) Yamada, N., Niwa, S., Tsujimura, T., Iwasaki, T., Sugihara, A., Futani, H., Hayashi, S., Okamura, H., Akedo, H. and Terada, N. Interleukin-18 and interleukin-12 synergistically inhibit osteoclastic bone-resorbing activity. Bone, 30, 901-908, 2002.

(80) Hosohara, K., Ueda, H., Kashiwamura, S., Yano, T., Ogura, T. Marukawa, S. and Okamura, H. Interleukin-18 induces acute biphasic reduction in the levels of circulating leukocytes in mice. Clin. Diagn. Lab. Immunol., 9, 777-783, 2002.

(81) Fujimori, Y., Yoshimoto, T., Matsui, K., Tsutsui, H., Okamoto, T., Kashiwamura, S., Hada, T., Okamura, H., Kakishita, E., Hara, H. and Nakanishi, K. Increased expression of interleukin-18 receptor on T lymphocytes in patients with acute graft-versus-host disease after allogeneic bone marrow transplantation. J. Interferon Cytokine Res., 22, 751-754, 2002.

(82) Naito, Y., Tsujino, T., Fujioka, Y., Ohyanagi, M., Okamura, H. and Iwasaki, T. Increased circulating interleukin-18 in patients with congestive heart failure. Heart, 88, 296-297, 2002.

(83) Tokmadzic, V.S., Tsuji, Y., Bogovic, T., Laskarin, G., Cupurdija, K., Strbo, N., Koyama, K., Okamura, H., Podack, E.R. and Rukavina, D. IL-18 is present at the maternal-fetal interface and enhances cytotoxic activity of decidual lymphocytes. Am. J. Reprod. Immunol., 48, 191-200, 2002.

(84) Yamashita K, Iwasaki T, Tsujimura T, Sugihara A, Yamada N, Ueda H, Okamura H, Futani H, Maruo S, and Terada N. Interleukin-18 inhibits lodging and subsequent growth of human multiple myeloma cells in the bone marrow. Oncol. Rep., 9, 1237-1244.

(85) Hayashi, H. Inoue, Y, Tsutsui H, Okamura H, Nakanishi K, Onozaki K. (2003) TGF-beta down-regulates IFN-gamma production in IL-18 treated NK cell line LNK5E6. Biochem. Biophys. Res. Commun., 300, 980-985, 2002.

(86) Hashimoto, W., Tanaka, F., Robbins, P.D., Taniguchi, M. and Okamura, H., Lotze, M.T. and Tahara, H. Natural killer, but not natural killer T, cells play a necessary role in the promotion of an innate antitumor response induced by IL-18. Int. J. Cancer, 103, 508-513, 2003.

(87) Kaneda, M., Kashiwamura, S., Ueda, H., Sawada, K., Sugihara, A., Terada, N., Kimura-Shimmyo, A., Fukuda, Y., Shimoyama, T. and Okamura, H. Inflammatory Liver Steatosis Caused by IL-12 and IL-18. J. Interferon Cytokine Res., 23, 155-162, 2003.

(88) Moriwaki, Y., Yamamoto, T., Shibutani, Y., Aoki, E., Tsutsumi, Z., Takahashi, S., Okamura, H., Koga, M., Fukuchi, M. and Hada, T. Elevated levels of interleukin-18 and tumor necrosis factor-alpha in serum of patients with type 2 diabetes mellitus: relationship with diabetic nephropathy. Metabolism, 52, 605-608, 2003.

(89) Kawasaki, D., Tsujino, T., Morimoto, S., Fujioka, Y., Naito, Y., Okumura, T., Masutani, M., Shimizu, H., Yuba, M., Ueda, A., Ohyanagi, M., Kashiwamura, S., Okamura, H. and Iwasaki, T. Usefulness of circulating interleukin-18 concentration in acute myocardial infarction as a risk factor for late restenosis after emergency coronary angioplasty. Am. J. Cardiol., 91, 1258-1261, 2003.

(90) Roles of caspase-1 in Listeria infection in mice. Tsuji, N.M., Tsutsui, H., Seki, E., Kuida, K., Okamura, H., Nakanishi, K. and Flavell, R.A. Int. Immunol., 16, 335-343, 2004.

(91) Okamoto, T., Yamada, N., Tsujimura, T., Sugihara, A., Nishizawa, Y., Ueda, H., Kashiwamura, S., Tsutsui, H., Futani, H., Maruo, S., Okamura, H. and Terada, N. Inhibition by interleukin-18 of the growth of Dunn osteosarcoma cells. J. Interferon Cytokine Res., 24,161-167, 2004.

(92) Oku, H., Tsuji, Y., Kashiwamura, S.I., Adachi, S., Kubota, A., Okamura, H. and Koyama, K. Role of IL-18 in pathogenesis of endometriosis. Hum. Reprod., 19, 709-714, 2004.

(93) Itoi, H., Fujimori, Y., Tsutsui, H., Matsui, K., Hada, T., Kakishita, E., Okamura, H., Hara, H. and Nakanishi, K. Differential upregulation of interleukin-18 receptor alpha chain between CD4+ and CD8+ T cells during acute graft-versus-host disease in mice. J. Interferon Cytokine Res., 24, 291-296, 2004.

(94) Kanno, T., Nagata, T., Yamamoto, S., Okamura, H. and Nishizaki, T. Interleukin-18 stimulates synaptically released glutamate and enhances postsynaptic AMPA receptor responses in the CA1 region of mouse hippocampal slices., Brain Res., 25,190-193, 2004.

(95) Itoi, H., Fujimori, Y., Tsutsui, H., Matsui, K., Sugihara, A., Terada, N., Hada, T., Kakishita, E., Okamura, H., Hara, H. and Nakanishi K. Involvement of interleukin-18 in acute graft-versus-host disease in mice. Transplantation, 15, 1245-1250, 2004.

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Cloning and expression of the rat interleukin-3 gene

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ABSTRACT

Genomic clones carrying the rat interleukin-3 (IL-3) gene have been isolated and the nucleotide sequence of the gene determined. Alignment of this sequence with that of the mouse IL-3 gene has allowed the structure of the rat IL-3 gene to be deduced. The intron-exon boundaries are conserved and extensive nucleotide homology (approx 90%) is present in the 5' flanking region and the portion of the gene coding for the signal peptide. Several proposed regulatory sequences are conserved and an analogous element to the tandem repeat in intron 2 of the mouse gene is also present. The predicted amino acid sequence for mature rat IL-3 shows surprisingly low homology (54%) with its murine counterpart, although all four cysteine residues are conserved. The rat IL-3 gene was expressed in monkey COS-1 cells and colony assays established that rat IL-3 is a multi-lineage haemopoietic growth regulator. There was little cross-reactivity of the respective IL-3 species on mouse and rat bone marrow cells suggesting that rat IL-3, in concert with its receptor, has evolved significantly away from the mouse IL-3/receptor system.

INTRODUCTION

Interleukin-3 (IL-3) is a member of the family of colony stimulating factors believed to regulate haemopoiesis[1-3]. It is involved in regulating growth and differentiation of pluripotent stem cells leading to the production of all the major blood cell types. The major natural source of IL-3 is the antigen-stimulated T lymphocyte, although it is also produced by a number of continuous cell lines[4-5]. IL-3 has been extensively characterized in the murine system, and cDNA[6,7] and genomic[8,9] clones have been reported recently. Little is known about IL-3 species in other mammals. There has been some biological characterization of mucosal mast cell growth factor in rats which may be equivalent to IL-3[10-13]. Pluripotent stem cell factor activity has been reported to be produced by the human bladder carcinoma cell line 5637[14], but it is still not established whether an exactly analogous lymphokine to murine IL-3 exists in man. Southern

hybridization analysis of mammalian DNA's, using a murine IL-3 cDNA probe, fails to detect homologous sequences in most mammalian species, even under conditions of relatively low stringency (unpublished data). This apparent low conservation of mammalian IL-3 genes contrasts with genes for other lymphokines such as IL-2, GM-CSF and Y-interferon which cross-hybridize between mouse and human[15-17].

As part of a study of the structure and biological role of IL-3 in other mammals, and in view of the possibility of studying the role of IL-3 using rat experimental models, we felt it was desirable to isolate the rat IL-3 gene and express it in animal cells. This is described in the present work together with the determination of some of the biological properties of recombinant rat IL-3.

MATERIALS AND METHODS

General Methods

(PVGxDA) male laboratory rats were used as the source of DNA for all experiments. Genomic DNA was prepared by homogenization and treatment with sarkosyl and proteinase K, followed by centrifugation in CsCl/ethidium bromide gradients[18,19]. Large scale λ DNA preparations were made from 1 litre cultures in Luria broth, lysed after 6 hr aeration at 37°C. Following addition of $CHCl_3$, phage DNA was purified as described[20]. Small scale preparations of λ DNA from plate lysates on Luria + Mg agarose plates were as described[8], and plasmid DNA minipreparations were made by the rapid boiling method[21]. Large scale plasmid preparations were carried out according to Clewell and Helinski[22] and plasmid DNA was purified using CsCl/ethidium bromide density gradients.

DNA fragments were isolated by electrophoretic separation on low-melting point agarose gels. Transformations were carried out using the high efficiency method of Hanahan[23].

The primers used for probe preparation and sequencing were synthesized by the phosphoramidite method. Autoradiography was carried out at -70°C using Fuji X-ray or Kodak XAR-5 film with Dupont Lightning Plus intensifying screens.

Construction and Screening of the Rat Genomic Library

Total genomic DNA from rat liver was partially digested with Sau3A and fragments in the size range 9-20kb were purified from low-melting point agarose. These fragments were ligated with λEMBL3A arms prepared by EcoRI/BamHI digestion of the phage DNA, and then packaged into bacteriophage particles[21,24]. Percentage recombinants was determined by spi selection[25].

The library was plated out for screening (using E.coli strain ED8655) on L top agarose/L agar in glass baking dishes (20cm x 30cm), approx 250,000 pfu per dish. Nitrocellulose replicas of plates containing plaques were prepared for hybridization as described by Benton and Davis[26].

Filters were routinely pre-washed in 50 mM Tris-HCl pH8/1M NaCl/1mMEDTA/0.1% SDS at 42°C for 1-2 hr, pre-hybridized in 6xSSC/5xDenhardt's solution/ 10mM EDTA/0.5% SDS/50μg/ml salmon sperm DNA at 65°C for 1-4 hr and hybridized in the same solution plus probe at 65°C for 18 hr. Filters were rinsed in 5xSSC/0.1%SDS and then washed twice in 2xSSC/0.1%SDS at 65°C for 45 min.

Probe Preparation

The 467 bp HindIII-NcoI fragment of the murine IL-3 cDNA-containing plasmid pILM3[6] was isolated and used as a template for primed synthesis of radioactively labelled cDNA, using a synthetic random decamer as primer. Probe preparation was as described[8], except that synthesized probe was routinely separated from un-incorporated label and very small labelled fragments by chromatography on Sephadex G-50 (fine).

Southern Hybridization

Restriction endonuclease digests of total genomic DNA (15μg per lane) or recombinant bacteriophage DNA (0.5-1μg per lane) were electrophoresed on 1% agarose/TAE (40mMTris-acetate/1mM EDTA) gels at 25mA for 16 hr. The gels were treated and blotted to nitrocellulose as described[27]. Pre-washing, pre-hybridization and hybridization of filters was as for genomic library screening. Following hybridization, filters were rinsed briefly in 5xSSC/0.1%SDS at room temperature, and then washed twice in 4xSSC/0.1%SDS at 65°C for 45 min. Stepwise reductions of salt

concentration (down to 0.1xSSC) were used for additional washes when necessary.

DNA Sequence Analysis

The sequence of the HindIII fragment covering the rat IL-3 gene was determined by the chain termination method of Sanger[28], using a universal flanking primer[29]. The DNA was prepared for sequencing by sonication of self-ligated fragment, T4 DNA polymerase repair and electrophoresis in low melting point agarose[30]. Fragments in size ranges 300-500 bp, 500-1000bp and 1000-2000bp were isolated from the gel and cloned into the SmaI site of M13mp10[31]. Competent cells of E.coli strain TG1, prepared by the method of Hanahan[23], were used for the transfection of the recombinant M13 DNA. Gels were routinely 5% acrylamide with "wedged" bottoms, and were dried to increase resolution[32]. Compressions were checked using gels containing 25% formamide[33]. DNA sequence data was entered directly from autoradiograms into the computer using a digitizer, and assembly and analysis of this data was performed using the computer programs of Staden[34-36]. The Genbank database was searched using the progam of Wilbur and Lipman[37].

Expression of Rat Interleukin-3

COS-1 cells were seeded at $5x10^5$ cells per 60mm diameter petri dish, grown overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), then transfected with the IL-3 expression vectors. 4 µg of plasmid DNA plus 16µg of carrier DNA was transfected per 10^6 cells by calcium phosphate precipitation and glycerol shock (15% glycerol for three minutes)[38,39]. The cells were washed in DMEM containing 10% FCS, and incubated in 5ml of the same medium at 37°C in 5% CO_2. Supernatants were collected at 72 and 144 hours post-transfection and filtered through Amicon 0.2µm filters. Samples were stored at 4°C prior to assay.

Assays for IL-3

1. Colony forming assays. Colony assays were done using bone marrow cells from WISTAR rats or BALB/c mice, scoring colonies on day 7 of culture[39]. The entire culture was fixed with 2.5% glutaraldehyde then stained with Lutol Fast Blue-hematoxylin to identify granulocyte-macrophage, erythroid, mixed erythroid and

megakaryocytic colonies. Each culture contained 75,000 bone marrow cells in 1.5 ml.

2. Bone marrow cell proliferation assays. Serial two-fold dilutions of test material were made in microtitre plates in 50µl RPMI 1640, 10% FCS. $5x10^4$ WISTAR rat or BALB/c mouse bone marrow cells were added to each well. Bone marrow cells from PVG, DA, PVG x DA, JC, PVG x JC and Fischer rats were also tested in this assay. Cultures were pulsed overnight on day 3 with [^{3}H]-thymidine. Activity is expressed as a reciprocal of the titration endpoint, the endpoint being defined as the supernatant dilution at which activity is no longer detectable[40].

RESULTS

Southern Hybridization of Rat Genomic DNA

Rat genomic DNA was digested with the restriction enzymes EcoRI and HindIII, electrophoresed on an agarose gel, blotted to nitrocellulose and hybridized with a murine IL-3 cDNA probe. A single hybridizing fragment of appropriate intensity for a single-copy gene was observed in the HindIII digest at approx. 5.8kb (Fig.1). The EcoRI digest gave two bands, suggesting the presence of an EcoRI site within the rat IL-3 gene. The reason for the different intensity of the two EcoRI bands is not clear but may be due to a greater representation of sequences derived from particular regions of the template fragment in the random primer probe.

Screening of the Rat Genomic Library

A library of approx. 10^6 recombinant phage was generated by cloning 9-20 kb fragments from a partial Sau3A digest of total genomic Rat DNA into λEMBL3A arms created by BamHI/EcoRI digestion. The complete library was plated out onto 4 baking dishes (approx. 250,000 recombinant phage per plate) and duplicate lifts were taken from each dish. A total of 5 plaques were identified by hybridization to the 467 bp HindIII-NcoI fragment of murine IL-3 cDNA labelled with ^{32}P by randomly primed synthesis. These 5 clones were purified and small scale λDNA preparations were carried out. Digestion of the DNA with EcoRI showed the five clones to be overlapping, each containing one or both of the two expected EcoRI fragments (Fig. 2). Clone λR3, which contained

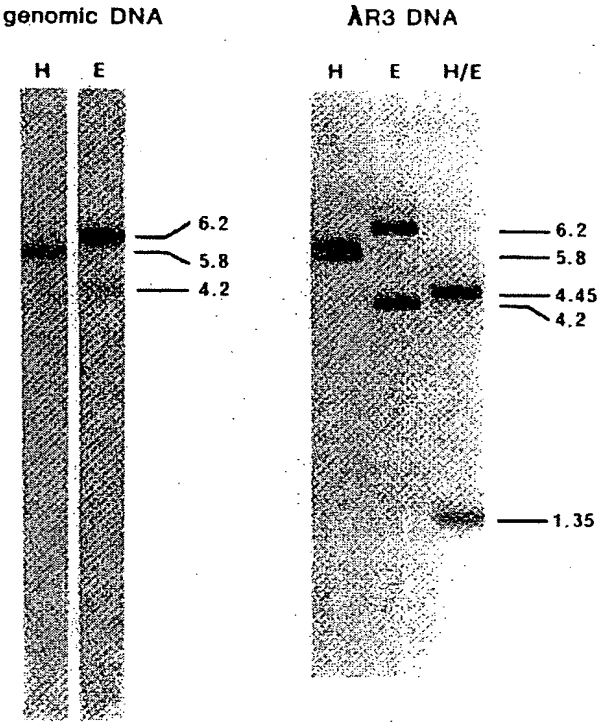


Figure 1 Southern blot analysis of rat genomic DNA and DNA from the genomic clone λR3, probed with an [α-^{32}P] dATP - labelled murine IL-3 cDNA fragment derived from pILM3[6]. Gel electrophoresis, blotting, probe preparation and hybridization conditions are given in Materials and Methods. Restriction endonuclease abbreviations are E, EcoRI and H, HindIII. Sizes given are in kilobase pairs (kb).

both fragments, was grown in large scale for further characterization. Southern hybridization of enzyme digests of λR3 DNA shows that it contains the fragments originally seen in the genomic DNA digests (Fig. 1) and allows localizaton of the rat IL-3 gene to a pair of fragments (3.3kb and 1.25kb) from a BamHI/HindIII double digest (Fig.2).

Nucleotide Sequence of the Rat IL-3 Gene

The nucleotide sequence of the 5.8 Kb HindIII fragment encompassing the rat IL-3 gene was determined on both strands by the chain termination method. The sequence of this region

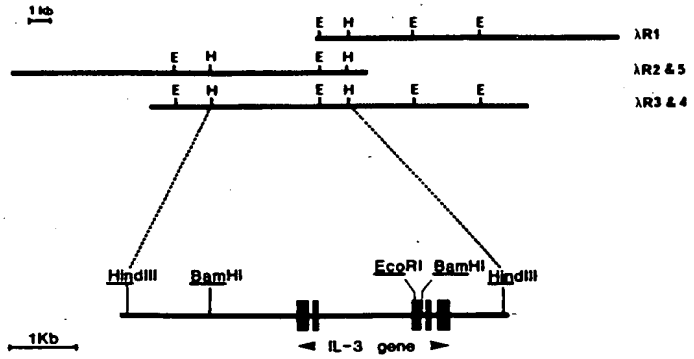


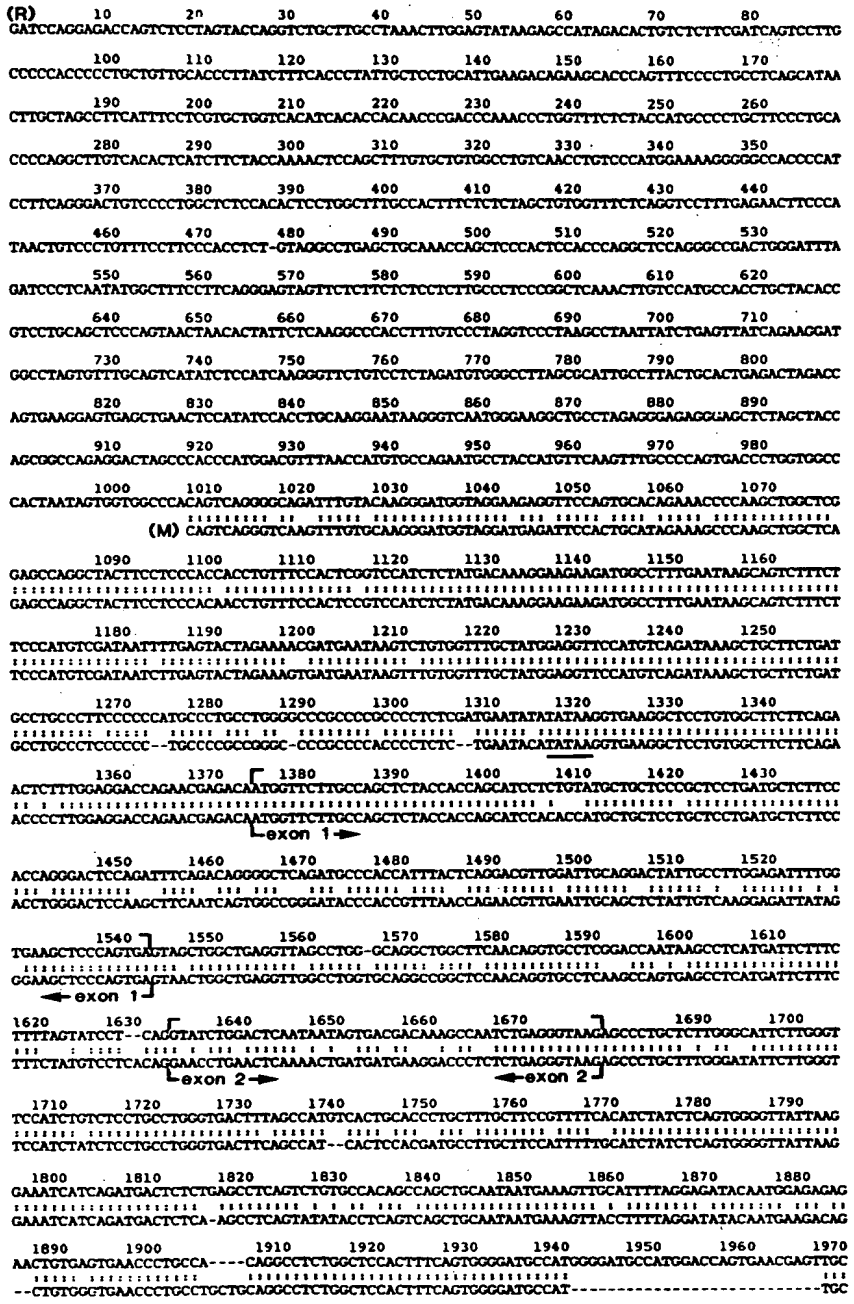
Figure 2 Restriction map of the rat IL-3 gene showing the regions covered by the 5 overlapping clones isolated from a λEMBL3A library of rat genomic DNA. The map of the gene was deduced from the characterization of purified λR3 DNA. Proposed exons are indicated by solid boxes, and cover the region from the AUG codon believed to be the start site of translation through to the translational termination codon UAA. Restriction endonuclease abbreviations are E, EcoRI and H, HindIII.

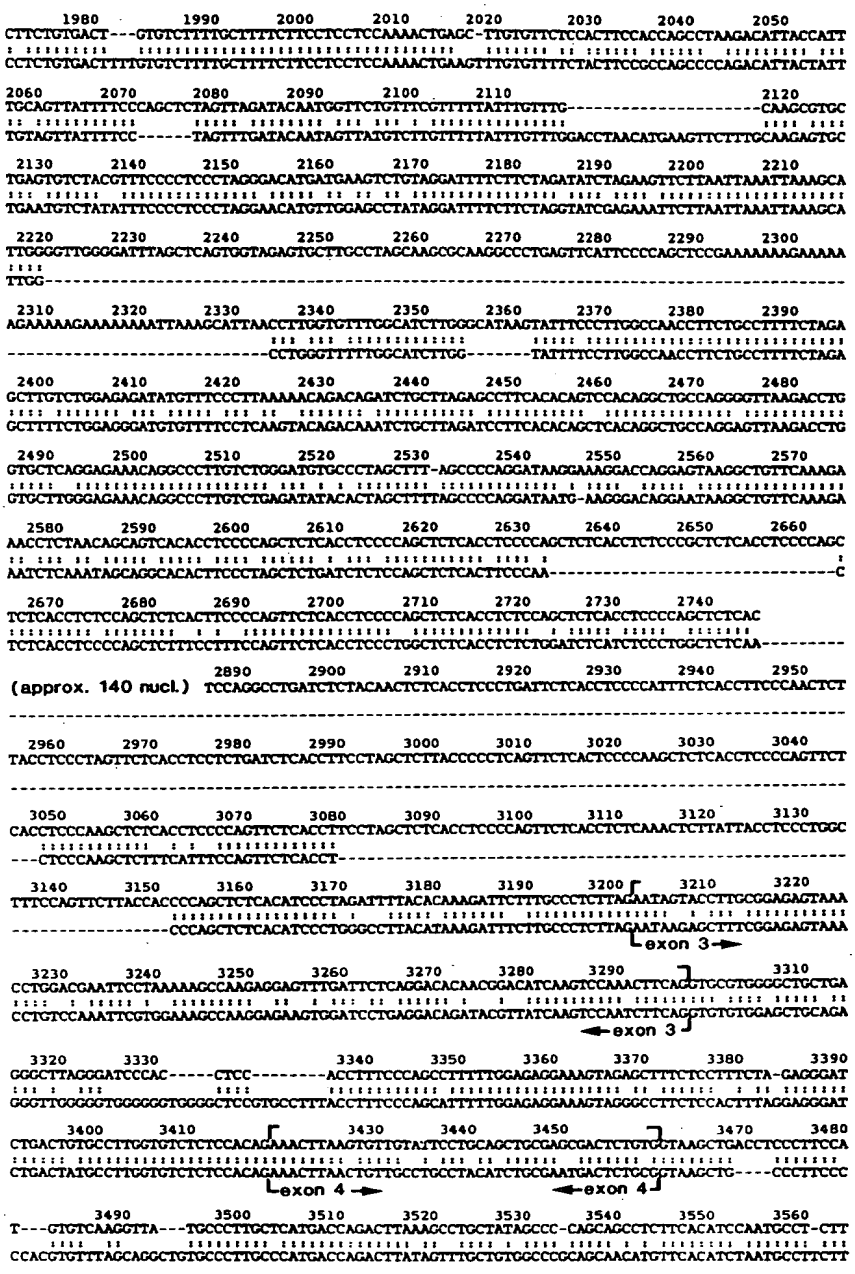
(excluding the 5' 1.25kb HindIII/BamHI fragment) is presented, together with an alignment against the murine IL-3 gene sequence, in Fig.3. The overall homology between the two nucleotide sequences in the coding regions is 76% ; the introns and flanking regions (approx. 200 nucleotides either side of the gene) are more highly conserved, with 80% and 90% homology respectively.

Structure of the Rat IL-3 Gene

Sufficient conservation of gene structure was present to allow the structure of the rat IL-3 gene to be deduced by alignment with the gene for murine IL-3 (Fig. 3). The rat IL-3 gene consists of five exons interrupted by four introns with conservation of the intron/exon boundaries between the rat and mouse genes. The deduction of the gene structure is greatly assisted by the high sequence homology through these regions. All introns interrupt the coding sequence between codons.

Sequence homology in the region corresponding to the leader sequence and extending 400 nucleotides upstream of the translational start site in murine IL-3 is extremely high. It is therefore likely that translation of rat IL-3 commences at nucleotide position 1374, making the first ten amino acids identical to those in the leader portion of murine IL-3. Transcriptional


(R)
(M)
exon 1
exon 1
exon 2
exon 2


(approx. 140 nucl.)
exon 3
exon 3
exon 4
exon 4

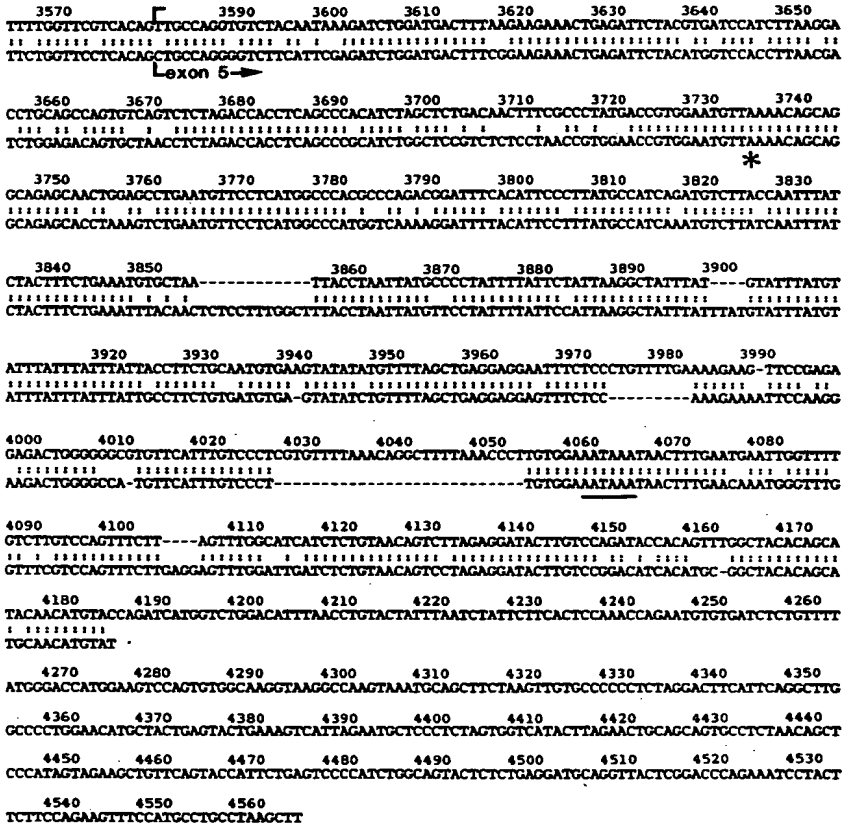


Figure 3 Nucleotide sequence of the rat (R) IL-3 gene covering the 3.3 Kb BamHI and 1.25 Kb BamHI/HindIII fragments, shown aligned against the sequence of the murine (M) IL-3 gene. TATA box and poly-adenylation signals are underlined. Intron/exon boundaries are shown as deduced for the murine gene[8,9].

control signals (including the potential enhancer hexanucleotide CCGCCC) described by Campbell et al[8] and Miyatake et al[9] are identical, although the proposed CAAT sequence would be a further 7 nucleotides upstream of the TATA box in the rat gene than is the case in murine IL-3.

Nucleotide sequence homology of the 3' end of the coding region is once again high, and there is an in-frame translational termination codon at position 3733. A single AATAAA sequence is found in the 3' untranslated region, 25 nucleotides nearer the

TAA stop codon than in murine IL-3. This sequence is likely to be involved in polyadenylation of the mRNA[41,42].

Other conserved features include two sets of tandemly repeated sequences. The first, a 14-15 bp element repeated about 12 times in intron 2 of murine IL-3, is also found in intron 2 of rat IL-3, except that in the latter case the repeat appears to extend for a further 400-450 nucleotides ie 25-30 more repeating units than in the murine gene. The complete sequence through these repeating units was not determined due to the difficulty in obtaining clones through this region. The amount of "missing" sequence was deduced by visual estimation from sequencing gel autoradiograms together with careful estimation of restriction fragment lengths. The consensus sequence of these repeats in the rat gene, AGCTCTCACCTCCC, is identical to the consensus sequence of the murine IL-3 intron 2 repeats[8]. The 9-bp inverted repeat which flanks these tandem repeats in the mouse IL-3 gene is not conserved in the rat gene. The second repetitive element occurs between the termination codon and the polyadenylation signal (nucleotides 3872-3922), and consists of nine copies of the sequence TATT[8].

One further feature of interest is the presence of sequences in the 5' untranslated region which show homology to murine B1, B2 and new Alu type repeating elements (data not shown). These elements represent major classes of short repeats dispersed throughout the mouse genome[43,44], and may each be present in up to 50,000 copies per haploid genome. Sequences homologous to rat poly (RY) repeats[45] are also found in this 5' region. The function of these sequences is unknown; there are suggestions ranging from a possible role in regulation of transcription/mRNA transport/translation[46-48] to the notion of "junk DNA" or pseudogenes[49].

Amino Acid Sequence of Rat IL-3

The deduced amino acid sequence of rat IL-3 is shown in Fig. 4 compared to murine IL-3. Overall homology is 59% which reduces to 54% in the "mature" protein ie. after the removal of the putative leader sequence[50,51], which is the most highly conserved part of the protein. Rat IL-3 has two potential N-glycosylation sites : the second of these corresponds to one of

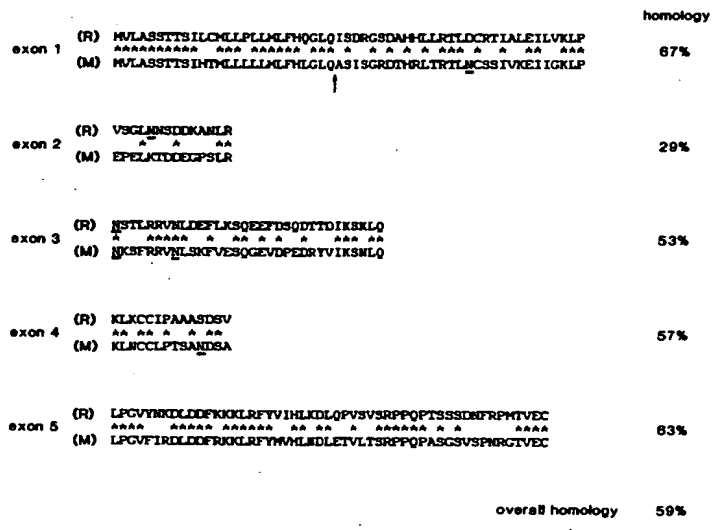


Figure 4 Comparison of predicted coding sequences of the exons of rat (R) and murine (M) IL-3 genes. Potential N-glycosylation sites are underlined. The arrow indicates the most likely site of cleavage of the signal peptide[50,51].

the 4 potential sites in murine IL-3. All four cysteine residues are conserved.

Expression of Rat IL-3 in Monkey COS-1 Cells

The 5.8kb HindIII fragment carrying the entire rat IL-3 gene (including transcriptional/translational control signals) was cloned into the HindIII site of the expression vector pSV2-neo[52]. Two clones were isolated, one for each possible orientation of the IL-3 gene relative to the SV40 promoter: pILR1 contained the IL-3 insert in the same orientation as the SV40 promoter and pILR2 had the insert in the reverse direction. Purified plasmid DNA from both constructions was used to transfect monkey COS-1 cells. Supernatants from the transfected cells were collected at 72 and 144 hours, based on previous experience with this expression system for murine IL-3. Murine IL-3 was prepared at the same time, using pSV2-neo vectors containing either genomic (pILM13) or cDNA (pILM4) murine IL-3 inserts. Supernatants from untransfected COS-1 cells were used as controls.

Colony Stimulating Activity of Rat IL-3

Recombinant rat IL-3 from both pILR1 and pILR2 stimulated

Table 1: Bone Marrow Proliferation Activity of Rat and Murine IL-3

Growth Factor	Source	Bone Marrow Proliferation[a] (Units/ml) Mouse	Rat
Mouse Il-3	WEHI-3 CM	1025	<10
Recombinant mouse IL-3	COS-1	250	<10
		360	<10
		90	<10
Recombinant rat IL-3	COS-1	<10	725
		10	510
		50	630
Mouse GM-CSF	purified[b]	3100	210

(a) Proliferation was measured as described in Methods using bone marrow cells from either Balb/c mice or WISTAR rats. Activity is expressed as the reciprocal of the titration end-point. COS-1 conditioned medium showed no detectable activity.
(b) Purified murine GM-CSF was obtained from Genzyme (Boston, Mass.) Lot No. 01531 and was verified to be GM-CSF by its ability to stimulate growth of FDC-P1 but not 32Dcl-23 cells.

colony formation only with rat bone marrow cells, while expressed murine IL-3 stimulated colony formation only with mouse cells. Bacterially expressed murine IL-3 was also inactive on the rat bone marrow cells.

In these experiments, rat IL-3 produced the full range of colony types expected for this factor on rat bone marrow cells, although there was a much greater proportion of megakaryocytes and eosinophils amongst the colonies generated than in the corresponding assays of murine IL-3 on murine cells. (A similar elevated response of megakaryocytes and eosinophils has been observed when assaying bacterially expressed murine IL-3). The biological properties of recombinant rat IL-3 will be reported in detail elsewhere (A.J.Hapel, D.R.Cohen and I.G.Young, In preparation).

Proliferation of Rat and Mouse Bone Marrow Cells in IL-3

Bone marrow cells are generally as sensitive as FDC-P1 cells for detecting IL-3 and GM-CSF activities in the mouse when used as at 10^5 cells per well in the standard microtitre cell proliferation assay. As there are no established cell line assays for

rat IL-3 we used bone marrow cells from a variety of rats and BALB/c mice to measure the growth factor activity of expressed rat and mouse IL-3.

The data in Table 1 show that rat IL-3 had good activity in promoting the proliferation of WISTAR rat bone marrow cells and slight activity on murine cells, while expressed murine IL-3 was active only on cells from murine bone marrow. This is in contrast to murine GM-CSF which has significant activity in the rat system. A similar result has been obtained using bone marrow cells from other strains of rats, except that PVG, PVG x JC and PVG x DA rats gave end-points about 4-8 fold greater than WISTAR, Fischer and JC rats. More extensive characterization of the biological relationship between rat and murine IL-3 will be described elsewhere (A.J.Hapel, D.R.Cohen and I.G.Young, In preparation).

DISCUSSION

Previous work on the biology and molecular biology of IL-3 has been predominantly with the mouse system. It is clear from the work described above that the rat genome carries an IL-3 gene which is closely related in overall structure to that found in the mouse genome. The cloning and sequence analysis of the rat IL-3 gene has enabled a detailed comparison to be made between the rat and mouse genes. The flanking regions (200 nucleotides either side of the gene) and introns of the mouse and rat IL-3 genes show 90% and 80% nucleotide homology, respectively, with the intron/exon junctions fully conserved. The portion of exon 1 encoding the putative signal peptide is also highly conserved (90%). Surprisingly, the nucleotide homology between the coding regions is only 76%. This is reflected in the amino acid homology which is only 54% for mature rat and mouse IL-3 (59% including the signal peptide). This is atypical of other lymphokines characterized to date. For example, γ-IFN shows an amino acid homology of 87% between mouse and rat[53], while IL-2 and GM-CSF show mouse : human amino acid homologies of 60% and 54% respectively[15,16,54]. The very high conservation of the signal peptide region observed between rat and mouse IL-3 is also not typical of the other lymphokines[15,16,53,54].

The conservation of the two sets of tandomly repeated units is also of interest. The TATT sequence, repeated nine times between the translational termination codon and the poly-adenylation signal in both murine and rat IL-3, is also found in a similar position in all IL-2, GM-CSF and Y-IFN genes sequenced to date[15-17,53-58]. The consensus sequence of the other repeat, a 14-15 bp element in intron 2, is identical to that of the tandem repeats in intron 2 of mouse IL-3. This element shows homology to a human BKV enhancer sequence homolog[8,59], and also to a portion of the consensus sequence for a 33-bp human myoglobin gene tandem repeat[8,60]. Similar repeats are found in the human insulin gene[61] and globin gene complex[62] and are associated with "hypervariable regions" of DNA.

Expression experiments using monkey COS-1 cells have demonstrated that the rat IL-3 gene encodes a multi-lineage haemopoietic growth regulator, which appears to have an analogous biological role to mouse IL-3[63]. The low amino acid homology between rat and mouse IL-3 correlates with the demonstration that rat and mouse IL-3 show little cross-reactivity. Other lymphokines and CSF's are somewhat varied in this regard. For example, human IL-2 is about 5 times more active on mouse cells than is murine IL-2 on human cells[15], whereas human and murine GM-CSF's do not cross-react either way[16]. Rat Y-IFN is active on mouse cells but not on human cells[53], and human M-CSF can stimulate growth of murine macrophages[64]. The low sequence homology and poor cross-reactivity suggest that rat IL-3, together with its receptor, have evolved significantly away from the murine IL-3/receptor system.

The availability of the rat IL-3 gene together with recombinant rat IL-3 should assist in studying the biological role of IL-3 in rat experimental models such as adjuvant-induced arthritis[65], graft rejection[66] and megakaryocyte proliferation[67,68]. The rat gene sequence also provides useful information for continuing studies on the regulatory elements controlling IL-3 expression.

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REFERENCES

1. Burgess, A.W., Camakaris, J. and Metcalf, D. (1977) J. Biol. Chem. 252, 1998-2003.
2. Howard, M., Burgess, A.W., McPhee, D. and Metcalf, D. (1980) Cell 18, 993-999.
3. Burgess, A.W., Metcalf, D., Russell, S.H.M. and Nicola, N.A. (1980) Biochem. J. 185, 301-314.
4. Ihle, J.N., Lee, J.C. and Rebar, L. (1981) J. Immun. 127, 2565-2570.
5. Ihle, J.N., Rebar, L., Keller, J., Lee, J.C. and Hapel, A.J. (1982) Immun. Rev. 63, 5-32.
6. Fung, M.C., Hapel, A.J., Ymer, S., Cohen, D.R., Johnson, R.M., Campbell, H.D. and Young, I.G. (1984) Nature 307, 233-237.
7. Yokota, T., Lee, F., Rennick, D., Hall, C., Arai, N., Mosmann, T., Nabel, G., Cantor, H. and Arai, K-I. (1984) Proc. Natl. Acad. Sci. USA 81, 1070-1074.
8. Campbell, H.D., Ymer, S., Fung, M.C. and Young, I.G. (1985) Eur. J. Biochem. 150, 297-304.
9. Miyatake, S., Yokota, T., Lee, F. and Arai, K-I. (1985) Proc. Natl. Acad. Sci. USA, 82, 316-320.
10. Haig, D.M., McKee, T.A., Jarrett, E.E.E., Woodbury, R. and Miller, H.R.P. (1982) Nature 300, 188-190.
11. Haig, D.M., McMenamin, C., Gunneberg, C., Woodbury, R. and Jarrett, E.E.E. (1983) Proc. Natl. Acad. Sci. USA 80, 4499-4503.
12. Abbud-Filho, M., Kupiec-Weglinski, J.W., Araujo, J.L., Heidecke, C.D., Tilney, N.L. and Strom, T.B. (1984) J. Immunol. 133, 2582-2586.
13. McMenamin, C., Jarrett, E.E.E. and Sanderson, A. (1985) Immunol. 55, 399-403.
14. Welte, K., Platzer, E., Lu, L., Gabrilove, J.L., Levi, E., Mertelsmann, R. and Moore, M.A.S. (1985) Proc. Natl. Acad. Sci. USA 82, 1526-1530.
15. Yokota, T., Arai, N., Lee, F., Rennick, D., Mosmann, T. and Arai, K-I. (1985) Proc. Natl. Acad. Sci. USA 82, 68-72.
16. Cantrell, M.A., Anderson, D., Cerretti, D.P., Price, V. McKereghan, K., Tushinski, R.J., Mochizui, D.Y., Larsen, A., Grabstein, K., Gillis, S. and Cosman, D. (1985) Proc. Natl. Acad. Sci. USA 82, 6250-6254.
17. Gray, P.W. and Goeddel, D.V. (1983) Proc. Natl. Acad. Sci. USA 80, 5842-5846.
18. Gross-Bellard, M., Oudet, P. and Chambon, P. (1973) Eur. J. Biochem. 36, 32-38.
19. Blin, N. and Stafford, D.W. (1976) Nucl. Acids Res. 3, 2303-2308.
20. Yamamoto, K.R., Alberts, B.M., Benzinger, R., Lawhorne, L. and Treiber, G. (1970) Virol. 40, 734-744.
21. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982)

Molecular Cloning: a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
22. Clewell, D.B. and Helinski, D.R. (1970) Biochem. 9, 4428-4440.
23. Hanahan, D. (1983) J. Mol. Biol. 166, 557-580.
24. Scalenghe, F., Turco, E., Edstrom, P., Pirrotta, V. and Melli, M. (1981) Chromosoma 82, 205-216.
25. Zissler, J., Singer, E. and Schaefer, F. (1971) in The bacteriophage lambda, ed. A.D. Hershey p.455. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
26. Benton, W.D. and Davis, R.W. (1977) Science 196, 180-182.
27. Meinkoth, J. and Wahl, G. (1984) Anal. Biochem. 138, 267-284.
28. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
29. Duckworth, M.L., Gait, M.J., Goelet, P., Hong, G.F., Singh, M. and Titmas, R.C. (1981) Nucl. Acids Res. 9, 1681-1706.
30. Deininger, P.L. (1983) Anal. Biochem. 129, 216-223.
31. Messing, J. (1983) Methods in Enzymol. 101, 20-78.
32. Garoff, H. and Ansorge, W. (1981) Anal. Biochem. 115, 450-457.
33. Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Peterson, G.F. (1982) J. Mol. Biol. 162, 729-773.
34. Staden, R. (1980) Nucl. Acids Res. 8, 3673-3694.
35. Staden, R. (1982) Nucl. Acids Res. 10, 4731-4751.
36. Staden, R. (1984) Nucl. Acids Res. 12, 499-503.
37. Wilbur, W.J. and Lipman, D.J. (1983) Proc. Natl. Acad. Sci. USA. 80, 726-730.
38. Parker, B.A. and Stark, G.R. (1979) J. Virol. 31, 360-369.
39. Hapel, A.J., Fung M.C., Johnson, R.M., Young, I.G., Johnson, G. and Metcalf D. (1985) Blood 65, 1453-1459.
40. Lafferty, K.J., Prowse, S.J., Al-Adra, A., Warren, H., Vasalli, J. and Reich, E. (1980) Aust. J. Exp. Biol. Med. Sci. 58,533-544.
41. Montell, C., Fischer, E.F., Caruthers, M.H. and Berk, A.J. (1983) Nature 305 600-605.
42. Nevins, J.R. (1983) Annu. Rev. Biochem. 52, 441-466.
43. Kramerov, D.A., Grigorian, A.A., Ryskov, A.P., and Georgiev, G.P. (1979) Nucl. Acids Res. 6, 697-713.
44. Kalb, V.F., Glasser, S., King, D. and Lingrel, J.B. (1983) Nucl. Acids Res. 11, 2177-2184.
45. Alonso, A., Kuehn, B. and Fischer, J. (1983) Gene 26, 303-306.
46. Thimmappaya, B., Weinberger, C., Schnieder, R.J. and Schenk, T. (1982) Cell 31, 543-551.
47. Walter, P. and Blobel, G. (1982) Nature 299, 691-698.
48. Carlson, D.P. and Ross, J. (1983) Cell 34, 857-864.
49. Kramerov, D.A., Tillib, S.V., Ryskov, A.P. and Georgiev, G.P. (1985) Nucl. Acids Res. 13, 6423-6437.
50. Conlon, P.J., Luk, K.H., Park, L.S., March, C.J., Hopp, T.P. and Urdal, D.L. (1985) J. Immunol. 135, 328-332.
51. Clark-Lewis, I., Kent, S.B.H. and Schrader, J.W. (1984) J. Biol Chem. 259, 7488-7494.
52. Southern, P.J. and Berg, P. (1982) J. Mol. Appl. Genet. 1, 327-341.
53. Dijkema, R., Van der Meide, P.H., Pouwels, P.H.,

Caspers, M., Dubbeld, M. and Schellekens, H. (1985) EMBO. J. 4, 761-767.
54. Kashima, N., Nishi-Takoaka, C., Fujita, T., Taki, S., Yamada, G., Hamuro, J. and Taniguchi, T. (1985) Nature 313, 402-404.
55. Gray, P.W. and Goeddel, D.V. (1982) Nature, 298, 859-863.
56. Gough, N.M., Gough, J., Metcalf, D., Kelso, A., Grail, D., Nicola, N.A., Burgess, A.W. and Dunn, A.R. (1984) Nature 309, 763-767.
57. Taniguchi, T., Matsui, H. Fujita, T., Takoaka, C., Kashima, N., Yoshimoto, R. and Hamuro, J. (1983) Nature 302, 305-310.
58. Fujita, T., Takoaka, C., Matsui, H. and Taniguchi, T. (1983) Proc. Natl. Acad. Sci. USA 80, 7437-7441.
59. Rosenthal, N., Kress, M., Gruss, P. and Khoury, G. (1983) Science 222, 749-755.
60. Jeffreys, A.J., Wilson, V. and Thein, S.L. (1985) Nature 314, 67-73.
61. Bell, G.I., Selby, M.J. and Rutter, W.J. (1982) Nature 295, 31-35.
62. Goodbourn, S.E.V., Higgs, D.R., Clegg, J.B. and Weatherall, D.J. (1983) Proc. Natl. Acad. Sci. USA 80, 5022-5026.
63. Iscove, N. and Roitsch, C. (1985) in Cellular and Molecular Biology of Lymphokines, ed. C. Sorg and A. Schimpl p.397-424. Academic Press, New York.
64. Stanley, E.R,. Chen, D-M. and Lin, H.S. (1978) Nature 274, 168-170.
65. Crowe, W.E., Battisto, J.R. and Smith, R.N. (1985) Arthritis Rheum. 28, 537-541.
66. Lowry, R.P. and Forbes, R.D.C. (1985) Transplant. Proc. 17, 1963-1965.
67. Ebbe, S. and Stohlman, F. Jr. (1965) Blood 26, 30-35.
68. Rolovic, Z., Jovanovic, T., Stankovic, Z. and Marinkovic, N. (1985) Blood 65, 60-64.

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Molecular Cloning and Expression of Rat Interleukin-1α cDNA

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A cDNA sequence coding for rat interleukin-1α (IL-1α) has been isolated from a cDNA library that was prepared with mRNA derived from LPS-stimulated rat peritoneal macrophages by using human IL-1α cDNA as a probe. The rat cDNA encodes a 270 amino acid residue protein which is homologous (65%) to human IL-1α. The rat cDNA sequence under SV40 early promoter directed the synthesis of biologically active IL-1 in monkey COS-1 cells. Rat IL-1α mRNA is not expressed in spleen, lung, liver or brain, and is also not expressed in these organs of LPS-treated rat except spleen. This suggests that IL-1α is not produced constitutively in various tissues and LPS is not sufficient to induce IL-1α in most tissues. Our data indicate that the IL-1 activities which have been reported to be produced in the brain are not of α type. We have constructed a plasmid expressing the carboxy terminal 156 amino acids in *Escherichia coli*. Recombinant rat IL-1α produced in COS cells or *E. coli* has cytotoxic activity against the human melanoma cell line A375S1 (GIF activity), which has been reported to be sensitive to human IL-1α and IL-1β. This suggests that GIF activity is common to IL-1s derived from various sources.

Interleukin-1 (IL-1) is a cytokine released from activated macrophages and numerous other cell types. It is involved in immunologic and inflammatory responses, including the stimulation of lymphocytes proliferation, lymphokine production, prostaglandin production, and the synthesis of acute phase proteins (*1*). It has become evident that IL-1 activities are encoded by two distinct cDNA sequences in human (*2-4*) and these cDNAs have been termed α and β, respectively. These cDNAs have been expressed in *E. coli*, and large amounts of both recombinant IL-1s have been purified by several groups (*5-11*). We have also described the isolation of both cDNAs from a cDNA library that was prepared with mRNA from human histiocytic lymphoma cell line U937 (*12*) and the purification of recombinant IL-1α and β expressed in *E. coli* (*13, 14*). The availability of recombinant IL-1 has permitted confirmation of the multiple biological activities of IL-1 (*14, 15*). Recently recombinant human IL-1 has been reported to stimulate the proliferation of astrocytes (*16*), the secretion of adrenocorticotropic hormone (ACTH) and cortisol in mice (*17*), the secretion of thyroid-stimulating hormone in rat pituitary cells (*18*), and the secretion of corticotropin-releasing factor in rats (*19, 20*). Thus, these reports indicate that IL-1 is also active in the regulation of the endocrine and central nervous systems. An animal model is required to elucidate the roles of IL-1 *in vivo*. The adjuvant arthritis rat (*21*), the glomerulonephritic rat (*22*), the adrenalectomized rat (*23*), and the hypophysectomized rat (*24*), among others, are very useful in this regard. However, the purification of rat IL-1, the preparation of antibody against rat IL-1, and the isolation of rat IL-1 cDNA have not been reported. So we first tried to isolate rat IL-1 cDNA. In this paper, we describe the molecular cloning of rat IL-1α cDNA, the expression of rat IL-1α mRNA in some tissues, and the production of the mature form of rat IL-1α in *E. coli*.

Abbreviations: IL-1, interleukin-1; LPS, lipopolysaccharide; PHA, phytohemagglutinin; FCS, fetal calf serum; PEC, peritoneal exudate cells; LAF, lymphocyte activating factor; GIF, growth inhibitory factor; ACTH, adrenocorticotropic hormone.

MATERIALS AND METHODS

Enzymes and Chemicals—Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd. Avian myeloblastosis virus reverse transcriptase was obtained from Bio-Rad Laboratories; calf thymus terminal deoxynucleotidyl transferase, *E. coli* DNA ligase and RNase H from Pharmacia; and *E. coli* DNA polymerase I from Boehringer Mannheim GmbH. All these enzymes were used according to the supplier's recommendations. [α-^{32}P]dCTP and [^{3}H]thymidine were purchased from Amersham. *E. coli* lipopolysaccharide (LPS) and proteose peptone were purchased from Difco Laboratories, phytohemagglutinin (PHA) from Burroughs-Wellcome, indomethacin from Kyowa Hakko Co., Ltd., oligo(dT) cellulose from Collaborative Research Inc., RPMI-1640 medium and fetal calf serum (FCS) from Gibco Laboratories, and Hanks' balanced salts from Flow Laboratories Inc. All of the other reagents used were analytical grade.

Isolation of RNA—Rat peritoneal exudate cells (PEC) were obtained according to the procedure described by Yamamoto and Tokunaga (*25*). Sprague-Dawley (SD) rats, purchased from Charles River Laboratories, were infused i.p. with 30 ml of 10% (w/v) proteose peptone. After 4 d, PEC were harvested by lavage with 20 ml of Hanks' balanced salts solution containing 20 u/ml heparin. They were washed twice with this solution and then with RPMI-1640 medium. The washed cells were resuspended at 2×10^6 cells/ml in RPMI-1640 medium supplemented with 10% FCS, 10 μg/ml LPS and 0.1 μg/ml indomethacin. PEC suspension (10 ml/dish) was adhered to plastic

culture dishes (90 mm diam.). After 13.5 h, the adhered cells were used for RNA preparation. Total cellular RNA was isolated by the guanidine thiocyanate/cesium chloride centrifugation method (*26*). Sprague-Dawley rats were injected intravenously with 100 μg/kg of LPS. After 6 h, total RNAs from tissues of rat were isolated by the guanidinium/hot phenol method (*27*) and then the guanidine thiocyanate/cesium chloride centrifugation method (*26*). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose column chromatography (*28*).

Cloning of Rat IL-1α cDNA—The oligo(dT)-tailed pcDV1 plasmid primer and the oligo(dG)-tailed pL1 linker fragment were prepared from plasmid pcDV1 and pL1, respectively, according to the methods of Okayama and Berg (*29, 30*). The cDNA library was constructed by using the above pcDV1 primer and pL1 linker fragment with poly(A)$^+$ RNA according to the procedure of Okayama and Berg (*30*). The human IL-1α cDNA insert (*Ball-MvaI* fragment, 285 bp) from plasmid pcD-GIF-207 (*12*) was radiolabeled with [α-^{32}P]dCTP by nick translation using the kit from Amersham and was used to screen 68,000 colonies of cDNA library. The hybridization solution was 20% formamide, 50 mM sodium phosphate (pH 6.5), 5× SSC (1×SSC=0.15 M sodium chloride, 0.015 M sodium citrate), 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. After overnight hybridization at 37°C, the filters were washed in 0.4×SSC and 0.1% SDS at 50°C. Two clones hybridized to the probe and were purified. The cDNA inserts to be analyzed were subcloned into plasmid pUC118 (*31*). A series of pUC118 subclones containing deleted cDNA inserts was prepared by the deletion kit for kilo-sequencing from Takara Shuzo Co., Ltd. based on the procedure of Henikoff (*32*) and Yanisch-Perron *et al.* (*33*) before sequencing by the dideoxynucleotides chain termination method (*34*).

Northern Blot Analysis—Poly(A)$^+$ RNA (5 μg) denatured with glyoxal was fractionated through 1.2% agarose gel and transferred to a nitrocellulose filter (*35*). The blot was hybridized with the radiolabeled rat IL-1α cDNA probe (*BalI-SacI* fragment, 1.6 kb) by nick translation. Hybridization was carried out at 42°C in 50% formamide, 5 × SSC, 50 mM sodium phosphate (pH 6.5), sonicated denatured salmon sperm DNA (100 μg/ml), and 0.08% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone. The filter was washed at 50°C in 0.1×SSC and 0.1% SDS.

Transfection into Monkey COS-1 Cells—The plasmid was transfected into monkey COS-1 cells as described previously (*12*).

Construction of pcDE Plasmid—The modified Okayama-Berg expression plasmid, pcDE, was constructed from pcDV1 and pL1. The pcDV1 (*30*) was digested with *KpnI* and treated with T4 DNA polymerase to convert the protruding 3′ ends to blunt ends. The blunt ends of the DNA were attached with *EcoRI* linker and digested with *EcoRI* and *HindIII*. The large fragment containing the polyadenylation signal sequence from the late region of SV40 DNA and pBR322 DNA segment was purified by agarose gel electrophoresis. The plasmid pL1 (*30*) was digested with *PstI* and both cohesive ends were converted to blunt ends with T4 DNA polymerase. The DNA was ligated with *EcoRI* linker and digested with *EcoRI* and *HindIII*. The small fragment, containing the SV40 early region promoter and two introns, was purified and ligated with the large fragment derived from the pcDV1 plasmid. The resulting plasmid, pcDE, has the single *EcoRI* site between the SV40 DNA segment containing early region promoter—two introns and the SV40 DNA segment containing the polyadenylation signal sequence.

Expression of Mature Rat IL-1α in E. coli—The expression plasmid ptrp-RT-IL-1α was constructed as shown in Fig. 4. The purified *HaeIII-AccI* fragment (530 bp) containing rat Il-1α cDNA was blunt-ended by the Klenow fragment of *E. coli* DNA polymerase I. It was inserted into the filled-in *HindIII* site of plasmid pTM1 (provided by Dr. Imamoto, unpublished data) containing the *E. coli trp* promoter-operator sequence to construct pTM1-RT-IL-1α(P). The *EcoRI-BamHI* fragment containing *E. coli trp* promoter-operator and rat IL-1α cDNA was inserted between the *EcoRI* and *BamHI* sites of pUC119 (*31*) to construct ptrp-RT-IL-1α(P). The single-stranded ptrp-RT-IL-1α(P) was prepared with the infection of the helper phage M13KO7 (*31*) and was annealed to the primer (5′) GGGTATCGATAATGTCAGCACCT(3′). The deletion of the extra cDNA and the insertion of the initiation codon ATG were carried out using the kit of the oligonucleotide-directed *in vitro* mutagenesis system from Amersham. The resulting double-stranded DNA was transformed into *E. coli* JM109 (*33*) to obtain ptrp-RT-IL-1α. *E. coli* JM109 harbouring ptrp-RT-IL-1α was grown for 9 h in M9 medium (*36*) supplemented with 1% casamino acids, 0.4% glucose, 5 μg/ml of thiamine-HCl, 20 μg/ml of L-cysteine, and 50 μg/ml of ampicillin. The *E. coli* cells were collected and lysed as described previously (*13*). Biological activity was measured for the *E. coli* cell extracts.

SDS-PAGE—SDS-PAGE was performed on 15% gel by the methods of Laemmli (*37*). The molecular weight standards used to calibrate the gel were: phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa, ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa. The gel was fixed and stained with Coomassie Brilliant Blue.

Biological Assays for Rat IL-1α—The assay for the growth inhibitory factor (GIF) against human melanoma cell line A375S1 has been described previously (*38*). The lymphocyte activating factor (LAF) activity was determined by measuring the incorporation of [^{3}H]thymidine into BALB/c mouse thymocytes in the presence of PHA according to the procedure described by Oppenheim *et al.* (*39*). One LAF unit is equal to half the value of maximum uptake of [^{3}H]thymidine with human recombinant IL-1β (*13*).

RESULTS

Isolation of cDNA Clones—Since activated macrophages produce IL-1, we prepared rat PEC elicited by proteose peptone. The LPS-stimulated adherent cells, a population

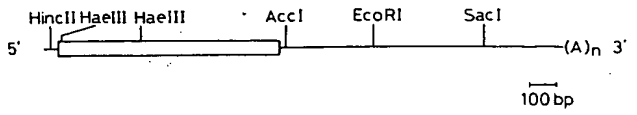

Fig. 1. **Restriction map of rat IL-1α cDNA (pcD-RT-IL-1α-6).** The coding region is shown as a box.

virtually of activated macrophages (*25*), were used as the source of mRNA. A cDNA library was constructed by using the Okayama-Berg cDNA expression vector. The probe was prepared from the 5′ end of human IL-1α cDNA (*BalI-MvaI*, 285 bp), whose nucleotide sequence is much more homologous to that of the 5′ end of murine IL-1α cDNA (*40*), and was used for the screening of the cDNA library under the condition of reduced stringency. Two clones were found to hybridize to the probe and were then purified. The cDNA inserts of the two clones were about 2.1 kb in length

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                                                                    AAAGTCTCCAGGGCACAGAGGGAGTCAACTCATTGGCGCTTGAGTCGGCAAAGAAATCAAG    61

ATG GCC AAA GTT CCT GAC TTG TTT GAA GAC CTA AAG AAC TGC TAT AGT GAA AAT GAA GAA   121
Met Ala Lys Val Pro Asp Leu Phe Glu Asp Leu Lys Asn Cys Tyr Ser Glu Asn Glu Glu
 1                                  10                                  20

TAC AGT TCT GCC ATT GAC CAT CTG TCT CTG AAT CAG AAA TCC TTC TAT GAT GCA AGC TAT   181
Tyr Ser Ser Ala Ile Asp His Leu Ser Leu Asn Gln Lys Ser Phe Tyr Asp Ala Ser Tyr
                                   30                                  40

GGC TCA CTT CAT GAG AAC TGC ACA GAT AAA TTT GTA TCT CTG AGA ACC TCT GAA ACA TCA   241
Gly Ser Leu His Glu Asn Cys Thr Asp Lys Phe Val Ser Leu Arg Thr Ser Glu Thr Ser
                                   50                                  60

AAG ATG TCC ACC TTC ACC TTC AAG GAG AGC CGG GTG GTG GTG TCA GCA ACA TCA AAC AAA   301
Lys Met Ser Thr Phe Thr Phe Lys Glu Ser Arg Val Val Val Ser Ala Thr Ser Asn Lys
                                   70                                  80

GGG AAG ATT CTG AAG AAG AGA CGG CTA AGT TTC AAT CAG CCC TTT ACT GAA GAT GAC CTG   361
Gly Lys Ile Leu Lys Lys Arg Arg Leu Ser Phe Asn Gln Pro Phe Thr Glu Asp Asp Leu
                                   90                                  100

GAG GCC ATA GCC CAT GAT TTA GAA GAG ACC ATC CAA CCC AGA TCA GCA CCT CAC AGC TTC   421
Glu Ala Ile Ala His Asp Leu Glu Glu Thr Ile Gln Pro Arg Ser Ala Pro His Ser Phe
                                  110                                 120

CAG AAT AAT TTG AGA TAC AAA TTG ATA AGG ATC GTC AAG CAG GAG TTC ATC ATG AAT GAT   481
Gln Asn Asn Leu Arg Tyr Lys Leu Ile Arg Ile Val Lys Gln Glu Phe Ile Met Asn Asp
                                  130                                 140

TCC CTC AAC CAA AAT ATA TAT GTG GAT ATG GAC AGA ATA CAT CTC AAA GCT GCT TCG TTA   541
Ser Leu Asn Gln Asn Ile Tyr Val Asp Met Asp Arg Ile His Leu Lys Ala Ala Ser Leu
                                  150                                 160

AAT GAC CTG CAG CTT GAA GTA AAA TTT GAC ATG TAT GCC TAC TCA TCG GGA GGA GAC GAC   601
Asn Asp Leu Gln Leu Glu Val Lys Phe Asp Met Tyr Ala Tyr Ser Ser Gly Gly Asp Asp
                                  170                                 180

TCT AAA TAT CCT GTG ACT CTC AAA GTC TCA AAT ACT CAG CTC TTT GTG AGT GCT CAG GGA   661
Ser Lys Tyr Pro Val Thr Leu Lys Val Ser Asn Thr Gln Leu Phe Val Ser Ala Gln Gly
                                  190                                 200

GAA GAC AAG CCT GTG TTG CTG AAG GAG ATT CCG GAA ACA CCA AAA CTC ATC ACA GGT AGT   721
Glu Asp Lys Pro Val Leu Leu Lys Glu Ile Pro Glu Thr Pro Lys Leu Ile Thr Gly Ser
                                  210                                 220

GAG ACC GAC CTC ATT TTC TTC TGG GAA AAA ATC AAC TCT AAG AAC TAC TTC ACA TCC GCA   781
Glu Thr Asp Leu Ile Phe Phe Trp Glu Lys Ile Asn Ser Lys Asn Tyr Phe Thr Ser Ala
                                  230                                 240

GCT TTC CCA GAG CTG TTA ATT GCC ACA AAA GAA CAA AGT CAG GTG CAC CTG GCA CGG GGA   841
Ala Phe Pro Glu Leu Leu Ile Ala Thr Lys Glu Gln Ser Gln Val His Leu Ala Arg Gly
                                  250                                 260

CTG CCC TCC ATG ATA GAT TTC CAG ATA TCA TAA AAACAGCCTTATTTAGGAGTCTACTTTCTTGAGAA   909
Leu Pro Ser Met Ile Asp Phe Gln Ile Ser
                                  270

GTGCTGACAATCTGTATGTACCATGTACAGGAAATGTTCCTCGTCCTAAGTCACTCGCATGGCATGTGCTGAGTCTTTG   988

TAATTCTAAATGAATGTTTACCCTCTTTGTAAGAGAAGAGCAAAGCCTAGTGGAACCAGCCCGACATATGATACTGTTT  1067

GTTATTTAAAGAGTACCCTATAGTTTGCTCACTACTAATCATTTTAATTATTATTCTTCATGGTATTCTTAGGAGGGTC  1146

AAAAAGACTCTGCTCATATTACAGATGGGCTAACTAAGGGATAAGACAACTGAAAAGTACACTCAATTGCATCTGGAAT  1225

GGGAATTCACAGACCCATGAAACTTATCTCACTGTGCACCTTCTGCTTCTAAAGTGCCGGATGGGTAGGATAAAGTTGT  1304

AAGAACGTAATGCTATCATTTTCAAAAGGAAGGGGACAATAGCTACATCTTTCCTACCTCAGTGGGTTTTATTCCAGTA  1383

AGAGCATTTGGATAAAATCCTTCTGTAACAGACCTCAAGAAGGAGACAGACTGTTGAATGTTATTTTTAAGTTATTTTA  1462

TATATGTATTTATAAATATATTTATGATAATTATATTATTTATGGCACATCCTTAAATCCTCTGAGCTTGCCAGGCATC  1541

CTCAGCAGCAGAATTTTCTAGGTGGTCAGTTAGATGCAGTGTCCTCTGAAACACCATGCTATAGACAGACGTACACTGT  1620

TTCCACATCCGTGGAGCTCTCTTTACATTCTTGTCCTTAGGAGCCCTGCAGTCATGATCACCAATCTGTACTGTTCACT  1699

TCGTTCATTTAAAATGATAACTGGGTCAGTCTTTTGCCCTCCTGTCCTTAAAGCTGTCTGGGTCTTCTTACATCATTCA  1778

ACTCACCTGTAATTAAAGCTAACCATCTAAAGACGGGAAGAACCTAACTGTGACAACCACATCCCTGTTACCTGAAGTT  1857

TCTTTTCTAGGATGTAATCAGTGTTTCCTCTGGGTTCCAAATTTGTTCTCAAACCAGGGTGTCACAAAAATATCAACAA  1936

TAATAATCAACTCATCATTACTATTGATCATAATTAAATAAAGCAAGTTTGAGCTGAAAAAAAAAAAAAAAAAAAAAAA  2015

AAAAAAAAAAAAAAAAA.....
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Fig. 2. **Nucleotide sequence and deduced amino acid sequence for the cDNA insert of clone pcD-RT-IL-1α-6.** Nucleotide numbers are shown along the right-hand side of the sequence and the deduced amino acid sequence and corresponding position numbers are shown beneath. The polyadenylation signal, AATAAA, is underlined.

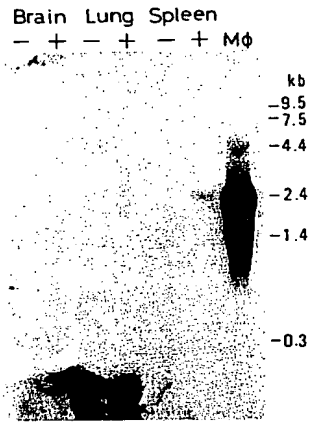

Fig. 3. **Northern blot analysis of rat IL-1α mRNA.** RNA was extracted from each tissue of rats injected without (−) or with (+) LPS. Mϕ represents RNA from LPS-stimulated rat peritoneal macrophages. Five micrograms of poly(A)$^+$ RNA was subjected to Northern blot analysis with the rat IL-1α cDNA probe.

TABLE I. **Expression of recombinant IL-1α in monkey COS-1 cells.** Individual plasmids were transfected into COS-1 cells using the DEAE-dextran method. After 72 h of incubation, the culture medium was harvested. The GIF activity and LAF activity in the culture supernatant were measured.

	GIF activity (u/ml)	LAF activity (u/ml)
pcD-RT-IL-1α-6	46.9	8
pcDE	0	0

and indistinguishable from each other on the basis of restriction map (Fig. 1). One of the clones, pcD-RT-IL-1α-6, was chosen for the analysis of the nucleotide sequences. As shown in Fig. 2, this cDNA insert is 1,992 bp in length excluding the poly(A)$^+$ tail flanking 14 bp 3′ to a typical poly(A) addition sequence (AATAAA) (*41*). Northern blot analysis showed that the rat IL-1α cDNA probe hybridized to a single-size mRNA of approximately 2.2 kb in the mRNA of LPS-stimulated rat peritoneal macrophages, which suggests that the size of cDNA insert is nearly full-length (Fig. 3).

The cDNA insert encodes a protein of 270 amino acid residues by the comparison of the rat cDNA with human IL-1α cDNA. The nucleotide sequence of the coding regions of this cDNA and human IL-1α cDNA is 73% homologous, while greater homology (78%) is found between the probe region of human IL-1α and the corresponding region of rat IL-1α. The amino acid sequence deduced from this cDNA sequence is homologous to human IL-1α (65%).

Expression of IL-1α mRNA in Rat—It is known that IL-1 is produced from various cell types stimulated with LPS (*1*). The expression of IL-1 mRNA in various tissues *in vivo*, however, has not been reported. We investigated the expression of IL-1α mRNA of brain, lung, spleen, and liver of rat treated or not treated with LPS. As shown in Fig. 3, the expression of rat IL-1α mRNA *in vivo* was not detected in the brain, spleen, lung, or liver of untreated rat (data not shown for liver). Rat IL-1α mRNA was expressed in small amounts in the spleen of LPS-treated rat, but not in the lung, brain, or liver (data not shown for liver).

Expression of Rat IL-1α in Monkey COS-1 Cells—To test the biological activities of the protein encoded in pcD-RT-IL-1α-6, it was transfected into monkey COS-1 cells, and the culture supernatant was assayed for IL-1 activity. The IL-1 activity was tested by measuring the LAF and GIF activities. As shown in Table I, the supernatant of cells transfected with pcD-RT-IL-1α-6 exhibited the LAF and GIF activities, while neither LAF nor GIF activity was found in the case of the control plasmid pcDE lacking the cDNA insert. These data confirm that the cDNA insert of pcD-RT-IL-1α-6 encodes rat IL-1α.

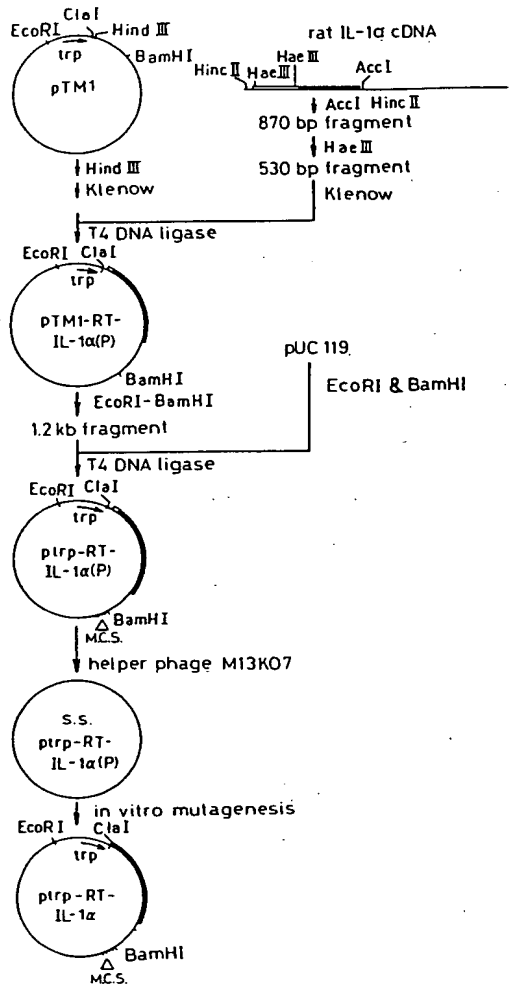

Fig. 4. **Construction of a plasmid expressing the mature form of rat IL-1α.** The construction is described in detail in "MATERIALS AND METHODS." The solid box on the rat IL-1α cDNA indicates the coding sequence for the mature protein while the open box indicates that for the precursor region. M.C.S., multiple cloning sites; s.s., single stranded.

Production of the Mature Form of Rat IL-1α in E. coli—Lomedico *et al.* have defined the amino-terminus of a subset of biologically active murine IL-1α at Ser^{115} (*40*). By matching the amino acid sequences of murine IL-1α and rat IL-1α shown in Fig. 6, it corresponds to Ser^{115} in the rat IL-1α sequence. We constructed the expression plasmid to direct the synthesis in *E. coli* of the carboxy terminal region encompassing the last 156 amino acids, beginning with the residue Ser^{115} of rat IL-1α, to prove the biological activity residing in the carboxy terminal region of rat IL-1α. The structure and the construction of the expression plasmid ptrp-RT-IL-1α is shown in Fig. 4. When the *E. coli* JM109 harbouring this plasmid was grown under conditions to derepress the tryptophan promoter, a large amount of rat

IL-1α mature protein was synthesized in *E. coli*, as shown in Fig. 5. The cell extract exhibited a high level of IL-1 activity, showing 5.6×10^6 units of GIF/ml of culture and 2×10^6 units of LAF/ml of culture. No IL-1 activity was found in cells transformed with the control plasmid pTM1 lacking the rat IL-1α sequence.

DISCUSSION

The cDNA of pcD-RT-IL-1α isolated with the 5′-end region of human IL-1α cDNA as the probe from the cDNA library prepared with mRNA derived from LPS-stimulated rat peritoneal macrophages encodes a protein which is homologous to human IL-1α in the amino acid sequence. It exhibited the GIF and LAF activities with the transfection into COS-1 cells. These data indicate that this cDNA encodes rat IL-1α.

The expression of IL-1α mRNA has been reported to be induced by LPS in peripheral blood monocytes (*3*) and with PMA and LPS in human histiocytic lymphoma cell line U937 (*42*). However, the level of IL-1α mRNA is lower than that of IL-1β mRNA induced simultaneously. On the other hand, keratinocytes have been demonstrated to contain more IL-1α mRNA than IL-1β mRNA in the absence of apparent stimulation (*43*). Our results shown in Fig. 3 indicate that IL-1α mRNA is not induced in normal rat tissues nor even in tissues of LPS-treated rat except the spleen, although IL-1α mRNA is induced in LPS-stimulated peritoneal macrophages. We have observed that IL-1β mRNA is induced in the brain, spleen, lung, and liver of LPS-treated rat (unpublished data). Therefore our data suggest that LPS is not sufficient to induce IL-1α mRNA in these organs, in contrast to monocytes.

Fontana *et al.* have reported that IL-1 activity was detected in the brain of LPS-treated mice (*44*). Giulian and Lachman have reported that IL-1 activity was detected in injured rat brain (*45*). Recently Farrar *et al.* have described that they detected IL-1β mRNA, but not IL-1α mRNA, by *in situ* hybridization throughout the rat brain, but they did not describe what species of IL-1 cDNA probe they used and they have not observed the expression of IL-1α mRNA in the brain of LPS-treated rat (*46*). The results of our experiment using rat IL-1α and β cDNA probes indicate that IL-1α mRNA is not expressed constitutively nor induced in the brain (Fig. 3), but IL-1β mRNA is induced in the brain of LPS-treated rat (unpublished results). Therefore IL-1 produced in the rat brain is thought to be the β type. It is important to understand the

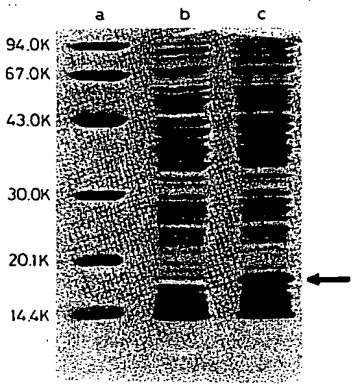

Fig. 5. **The expression of recombinant rat IL-1α on SDS-PAGE.** *E. coli* cells grown for 9 h under the derepression of the trp promoter were collected and dissolved in Laemmli sample buffer (*37*) and electrophoresed in 15% polyacrylamide gel. The arrow shows the mature form of rat IL-1α produced in *E. coli*. Lane 1, molecular weight markers; lane 2, total protein from *E. coli* JM109/pTM1 (without rat IL-1α cDNA); lane 3, total protein from *E. coli* JM109/ptrp-RT-IL-1α (with rat IL-1α cDNA).

```
Rat      MAKVPDLFEDLKNCYSENEEYSSAIDHLSLNQKSFYDASYGSLHENCTDK   50
Murine   ····················D···························T···Q   50
Rabbit   ··············F·····························EP···D·MN·   50
Human    ·····M·····························D··S·····HV···P···G·M·Q   50

Rat      FVSLRTSETSKMSTFTFKESRVVVSATSNKGKILKKRRLSFNQPFTEDDL  100
Murine   ·············N·····T·····SN··········SET·····      100
Rabbit   V···S·····VSPNL··Q·NV·A·T·---S··········L···I·DV··   97
Human    S···SI·····T·KL·····M···AT---N··V·····LS·SI·D···    97
                          *
Rat      EAIAHDLEE-TIQPRSAPHSFQNNLRYKLIRIVKQEFIMNDSLNQNIYVD  149
Murine   QS·T·····-·····YTY·SD·····MKL·R·K·V·····T··Q·       149
Rabbit   ·TNVS·P··GI·K···V·YT··R·M···YL··I····TL··A···SLVR·  147
Human    ····N·S··EI·K·····F··LS·VK·NFM··I·Y···L··A···S·IR-  146
                  ▼                                  ▼
Rat      MDRIHLKAASLNDLQLEVKFDMYAYSSGG-DDSKYPVTLKVSNTQLFVSA  198
Murine   V·KHY·STTW·····Q·····-·····I·DS·····                 198
Rabbit   TSDQY·Q··P·QN·GDA·····GV··-MTS-E··IL····RI·Q·P·····  195
Human    ANDQY·T··A·HN·DEA·····G···-KSSK··A·IT·I·RI·K···Y·T·  195
                                                          ▼
Rat      QGEDKPVLLKEIPETPKLITGSETDLIFFWEKINSKNYFTSAAFPELLIA  248
Murine   ····Q·····L·····KS·····Y···F··                       248
Rabbit   ·N··E·····M····RI··D··S·IL····TQGN····K···N·Q·F··   245
Human    ·D··Q·····M··I··T·····N·L····THGT·····V·H·N·F··     245
                       ▼
Rat      TKEQSQVHLARGLPSMIDFQIS                               270
Murine   ·····R·····T·····                                    270
Rabbit   ··PEHL··M·····T·····                                 267
Human    ··QDYW·C··G·P··IT····LENQA                           271
```

Fig. 6. **The comparison of the amino acid sequence of rat IL-1α with IL-1α sequences from murine (*40*), rabbit (*4*), and human(*3, 4, 12*).** The letters represent the single-letter abbreviations for amino acids. The dots represent an amino acid residue that is identical to the residue shown for rat IL-1α. The asterisk shows the putative amino terminal amino acid residue of the mature form of rat IL-1α. The closed triangles indicate amino acid residues which are conserved among murine, rabbit, and human, but not in rat. Gaps (−) have been inserted to achieve maximum homology.

difference of the expression of IL-1α and β mRNAs in the brain. Uehara *et al.* have reported that human recombinant IL-1β increased the plasma levels of ACTH in rats, whereas human recombinant IL-1α did not (*47*). It has also been demonstrated that recombinant human IL-1β increased the ACTH content of the anterior pituitary and corticotropin releasing hormone (CRH) content of the hypothalamus and adrenal glands in rats, but recombinant human IL-1α had no such effects (Y. Naitoh *et al.*, submitted). It is interesting that their data suggest the difference of the function between IL-1α and IL-1β in the brain, which might relate to the difference of expression of IL-1α and β mRNAs in the brain. The availability of recombinant rat IL-1α will contribute to confirming their data.

The comparison of the amino acid sequence deduced from rat IL-1α cDNA with those of murine, rabbit, and human IL-1αs is shown in Fig. 6. In amino acid sequence, rat IL-1α is homologous to human IL-1α (65%), rabbit IL-1α (64%) (*4*), and murine IL-1α (83%).

When the amino acid sequence of rat IL-1α is divided into the precursor region (Met^{1}-Arg^{114}) and the mature region (Ser^{115}-Ser^{270}), the precursor region is much more homologous to human IL-1α (74%), rabbit IL-1α (71%), and murine IL-1α (89%). We have no idea of the significance of the greater conservation of the precursor region than of the mature region in IL-1α. Recently it has been shown that both the precursor form and the mature form of human IL-1α bind to the IL-1 receptor (*48*) and that membrane-associated, biologically active IL-1 is α type, not β type (*49*). These functions of IL-1α might relate to the conservation of the precursor region in IL-1α.

There is another feature of interest in the amino acid sequence data: at four sites the same residue is found in the murine, rabbit, and human sequences, whereas there is a change in the rat gene (Fig. 6). This is due to the substitution of one nucleotide in the codon between rat and murine. These amino acid residues lie in the mature region of rat IL-1α and hence might not be essential for the biological activity.

The rat IL-1α, encoded in the cDNA, exhibited the IL-1 activity upon the transfection into COS-1 cells, but its amino terminal amino acid has not been determined. However, the mature rat IL-1α beginning at Ser^{115} expressed in *E. coli* exhibited the IL-1 activity. This recombinant IL-1α from *E. coli* has been purified. Its specific activity is about 1×10^{7} GIF units/mg protein and its amino terminus is a serine residue (Y. Masui *et al.*, unpublished results), which indicates that the carboxy terminal region of rat IL-1α is sufficient for the IL-1 activity, as reported for murine IL-1α (*40*) and human IL-1α (*3, 14*).

We measured the LAF activity as IL-1 activity. We also measured the GIF activity since recombinant human IL-1 α and β are known to inhibit the growth of human melanoma cell line A375 (*12, 38, 50*). Recombinant rat IL-1α has also the GIF activity, suggesting that GIF activity is common to IL-1s derived from various sources.

We have already obtained the recombinant rat IL-1α and the antibody against rat IL-1α. These materials and the cDNA will contribute to clarifying the regulation of gene expression and the biological activities of IL-1 *in vivo*.

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REFERENCES

1. Oppenheim, J.J., Kovacs, E.J., Matsushima, K., & Durum, S.K. (1986) *Immunol. Today* 7, 45-56
2. Auron, P.E., Webb, A.C., Rosenwasser, L.J., Mucci, S.F., Rich, A., Wolff, S.M., & Dinarello, C.A.(1984) *Proc. Natl. Acad. Sci. U.S.* 81, 7907-7911
3. March, C.J., Mosley, B., Larsen, A., Cerretti, D.P., Braedt, G., Price, V., Gillis, S., Henney, C.S., Kronheim, S.R., Grabstein, K., Conlon, P.J., Hopp, T.P., & Cosman, D. (1985) *Nature* 315, 641-647
4. Furutani, Y., Notake, M., Yamayoshi, M., Yamagishi, J., Nomura, H., Ohue, M., Furuta, T., Fukui, T., Yamada, M., & Nakamura, S. (1985) *Nucleic Acids Res.* 13, 5869-5882
5. Kronheim, S.R., Cantrell, M.A., Deeley, M.C., March, C.J., Glackin, P.J., Anderson, D.M., Hemenway, T., Merriam, J.E., Cosman, D., & Hopp, T.P. (1986) *Bio/Technol.* 4, 1078-1082
6. Gubler, U., Chua, A.O., Stern, A.S., Hellmann, C.P., Vitek, M.P., Dechiara, T.M., Benjamin, W.R., Collier, K.J., Dukovich, M., Familletti, P.C., Fiedler-Nagy, C., Jenson, J., Kaffka, K., Kilian, P.L., Stremlo, D., Wittreich, B.H., Woehle, D., Mizel, S.B., & Lomedico, P.T. (1986) *J. Immunol.* 136, 2492-2497
7. Wingfield, P., Payton, M., Tavernier, J., Barnes, M., Shaw, A., Rose, K., Simona, M.G., Demczuk, S., Williamson, K., & Dayer, J.-M. (1986) *Eur. J. Biochem.* 160, 491-497
8. Dinarello, C.A., Cannon, J.G., Mier, J.W., Bernheim, H.A., Lopreste, G., Lynn, D.L., Love, R.N., Webb, A.G., Auron, P.E., Reuben, R.C., Rich, A., Wolff, S.M., & Putney, S.D. (1986) *J. Clin. Invest.* 77, 1734-1739
9. Tocci, M.J., Hutchinson, N.I., Cameron, P.M., Kirk, K.E., Norman, D.J., Chin, J., Rupp, E.A., Limjuco, G.A., Bonilla-Argudo, V.M., & Schmidt, J.A. (1987) *J. Immunol.* 138, 1109-1114
10. Meyers, C.A., Johanson, K.O., Miles, L.M., McDevitt, P.J., Simon, P.L., Lee Webb, R., Chen, M.-J., Holskin, B.P., Lillquist, J.S., & Young, P.R. (1987) *J. Biol. Chem.* 262, 11176-11181
11. Wingfield, P., Payton, M., Graber, P., Rose, K., Dayer, J.-M., Shaw, A.R., & Schmeissner, U. (1987) *Eur. J. Biochem.* 165, 537-541
12. Nishida, T., Nishino, N., Takano, M., Kawai, K., Bando, K., Masui, Y. Nakai, S., & Hirai, Y. (1987) *Biochem. Biophys. Res. Commun.* 143, 345-352
13. Kikumoto, Y., Hong, Y.-M., Nishida, T., Nakai, S., Masui, Y., & Hirai, Y. (1987) *Biochem. Biophys. Res. Commun.* 147, 315-321
14. Hirai, Y., Masui, Y., Nakai, S., Kikumoto, Y., Nishida, T., & Hong, Y.-M. (1988) in *Gann Monograph on Cancer Research* (Hashimoto, Y. & Hamaoka, T., eds.) Vol. 34, pp. 155-166, Japan Scientific Societies Press, Tokyo
15. Dinarello, C.A. (1986) *Immunobiology* 172, 301-315
16. Lachman, L.B., Brown, D.C., & Dinarello, C.A. (1987) *J. Immunol.* 138, 2913-2916
17. Besedovsky, H., del Rey, A., Sorkin, E., & Dinarello, C.A. (1986) *Science* 233, 652-654
18. Bernton, E.W., Beach, J.E., Holaday, J.W., Smallridge, R.C., & Fein, H.G. (1987) *Science* 238, 519-521
19. Sapolsky, R., Rivier, C., Yamamoto, G., Plotsky, P., & Vale, W. (1987) *Science* 238, 522-524
20. Berkenbosch, F., van Oers, J., del Rey, A., Tilders, F., & Besedovsky, H. (1987) *Science* 238, 524-526
21. Rosenthale, M.E. & Capetola, R.J. (1982) *Fed. Proc.* 41, 2577-2582
22. Werber, H.I., Emancipator, S.N., Tykocinski, M.L., & Sedor, J.R. (1987) *J. Immunol.* 138, 3207-3212
23. Thompson, W.L., Abeles, F.B., Beall, F.A., Dinterman, R.E., & Wannemacher, R.W., Jr. (1976) *Biochem. J.* 156, 25-32
24. Kampschmidt, R.F., Upchurch, H.F., & Pulliam, L.A. (1973) *Proc. Soc. Exp. Biol. Med.* 143, 279-283
25. Yamamoto, S. & Tokunaga, T. (1978) *Microbiol. Immunol.* 22,

27-40
26. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., & Rutter, W.J. (1979) *Biochemistry* **18**, 5294-5299
27. Maniatis, T., Fritsch, E.F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* pp. 194-195, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.* **69**, 1408-1412
29. Okayama, H. & Berg, P. (1982) *Mol. Cell. Biol.* **2**, 161-170
30. Okayama, H. & Berg, P. (1983) *Mol. Cell. Biol.* **3**, 280-289
31. Vieira, J. & Messing, J. (1987) in *Methods in Enzymology* (Wu, R. & Grossman, L., eds.) Vol. 153, pp. 3-11, Academic Press, New York
32. Henikoff, S. (1984) *Gene* **28**, 351-359
33. Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene* **33**, 103-119
34. Sanger, F., Nicklen, S., & Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. U.S.* **74**, 5463-5467
35. Thomas, P.S. (1980) *Proc. Natl. Acad. Sci. U.S.* **77**, 5201-5205
36. Miller, J.H. (1972) *Experiments in Molecular Genetics* p. 431, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. Laemmli, U.K. (1970) *Nature* **227**, 680-685
38. Nakai, S., Mizuno, K., Kaneta, M., & Hirai, Y. (1988) *Biochem. Biophys. Res. Commun.* **154**, 1189-1196
39. Oppenheim, J.J., Shneyour, A., & Kook, A.I. (1976) *J. Immunol.* **116**, 1466-1472
40. Lomedico, P.T., Gubler, U., Hellmann, C.P., Dukovich, M., Giri, J.G., Pan, Y.-C.E., Collier, K., Semionow, R., Chua, A.O., & Mizel, S.B. (1984) *Nature* **312**, 458-461
41. Proudfoot, N.J. & Brownlee, G.G. (1976) *Nature* **263**, 211-214
42. Nishida, T., Takano, M., Kawakami, T., Nishino, N., Nakai, S., & Hirai, Y. (1988) *Biochem. Biophys. Res. Commun.* **156**, 269-274
43. Kupper, T.S., Ballard, D.W., Chua, A.O., McGuire, J.S., Flood, P.M., Horowitz, M.C., Langdon, R., Lightfoot, L., & Gubler, U. (1986) *J. Exp. Med.* **164**, 2095-2100
44. Fontana, A., Weber, E., & Dayer, J.-M. (1984) *J. Immunol.* **133**, 1696-1698
45. Giulian, D. & Lachman, L.B. (1985) *Science* **228**, 497-499
46. Farrar, W.L., Hill, J.M., Harel-Bellan, A., & Vinocour, M. (1987) *Immunol. Rev.* **100**, 361-378
47. Uehara, A., Gottschall, P.E., Dahl, R.R., & Arimura, A. (1987) *Biochem. Biophys. Res. Commun.* **146**, 1286-1290
48. Mosley, B., Urdal, D.L., Prickett, K.S., Larsen, A., Cosman, D., Conlon, P.J., Gillis, S., & Dower, S.K. (1987) *J. Biol. Chem.* **262**, 2941-2944
49. Conlon, P.J., Grabstein, K.H., Alpert, A., Prickett, K.S., Hopp, T.P., & Gillis, S. (1987) *J. Immunol.* **139**, 98-102
50. Lachman, L.B., Dinarello, C.A., Llansa, N.D., & Fidler, I.J. (1986) *J. Immunol.* **136**, 3098-3102

THE RAT INTERLEUKIN-5 GENE: CHARACTERIZATION AND EXPRESSION BY RETROVIRAL GENE TRANSFER AND POLYMERASE CHAIN REACTION

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The rat interleukin-5 (IL-5) gene was isolated from a genomic lambda phage library and a fragment containing all four exons was inserted into the retroviral vector pXT1, resulting in pXTRIL5. Upon retroviral gene transfer into two IL-5–dependent mouse cell lines, B13 and T88M, autonomously growing cells were established and B-cell growth factor activity was detected in the supernatants of the infected cells. "cDNA" versions of the rat IL-5 gene were rescued by the polymerase chain reaction (PCR) with primers specific for the flanking regions of the cloning site in pXT1. Restriction or DNA sequence analysis of five different clones revealed precise splicing in two cases, while three of the clones had retained the first intron. In addition, in two of these about 400 bp of rat IL-5 5′ flanking regions were deleted. The sequence comparison of rat, mouse, and human IL-5 genes revealed a high degree of conservation (e.g., mouse and rat were 92% homologous at the amino acid level). The combination of retroviral gene transfer and PCR may offer an alternative, efficient method for the cloning of cDNAs.

Interleukin-5 (IL-5) was originally described as B-cell growth factor (BCGF) or T-cell replacing factor (TRF).[1,2] Mouse and human IL-5 genes have been isolated and characterized at both the cDNA and the genomic level.[3-6] Comparison of rat and mouse interleukin-4 (IL4) genes recently revealed an unusual high degree of divergence.[7] Both interleukins, IL-5 and IL-4, are synthesized simultaneously and predominantly by TH2 cells[8] and their genes are located close to each other on the same chromosome.[9,10] Both lymphokines stimulate growth and affect differentiation of B cells.[11] In this report we wish to extend our comparative study of lymphokine genes. In order to circumvent conventional cloning of the rat IL-5 cDNA we chose an alternative approach. Shimotohno and Temin[12] reported the loss of intervening sequences from a retroviral vector upon retroviral passage. Subsequently, a number of groups demonstrated the possibility of generating a "cDNA" of genomic genes through retroviral gene transfer.[13-18] The rescue of such "cDNAs" was hampered by undetectable gene expression,[19] by inefficient or irregular splicing,[13,16,19] and by time-consuming and laborious protocols.[13,15-17] Therefore, this approach did not seem to be useful for the generation of cDNAs of unknown genes. The introduction of the polymerase chain reaction (PCR) technique into molecular biology[20] has facilitated the analysis of large numbers of different proviral integrations. The detection of completely spliced cDNAs should be possible, even without knowledge of the sequence which had been cloned into the retroviral vector. Using the combination of retroviral gene transfer and PCR we report here the isolation and characterization of the rat IL-5 "cDNA."

RESULTS

Identification and Isolation of the Rat IL-5 Gene

EcoRI- and BamHI-digested liver DNA of Lewis rat and BALB/c mouse were analysed by Southern blot hybridization using a mouse IL-5 cDNA probe (Fig. 1). A single hybridizing EcoRI fragment of 8 kb and two BamHI fragments were obtained from rat DNA, which

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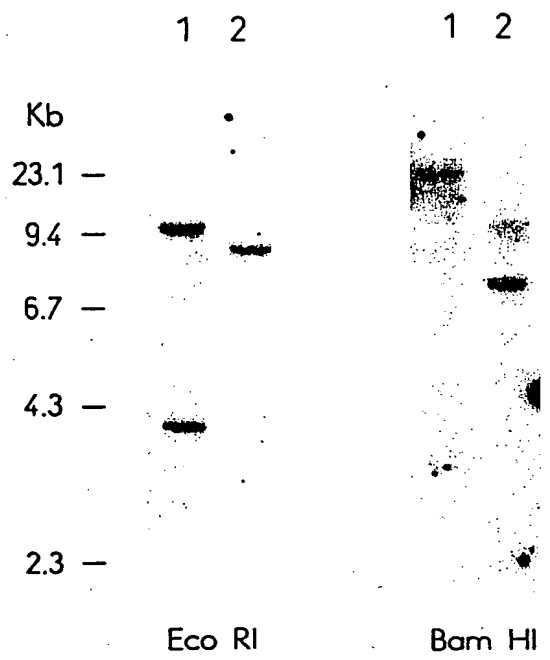

Figure 1. Southern blot analysis of BALB/c mouse (1) and Lewis rat (2) liver DNA.

EcoRI- and BamHI-digested DNAs were hybridized with the 630-bp BamHI-AccI mouse IL-5 cDNA fragment of pSP6K-mTRF23. Size marker was HindIII-digested lambda DNA.

is consistent with a single copy gene in the rat genome. Similar hybridization intensities in comparison to BALB/c indicated a high degree of homology between rat and mouse IL-5 genes. A genomic library was constructed with Lewis rat liver DNA, partially digested by Sau3A. Recombinant plaques (1×10^6) were screened with a mouse IL-5 cDNA probe. A clone was isolated which contained the 8-kb IL-5–specific EcoRI fragment. Further characterization by restriction and Southern blot analysis with mouse exon-specific probes indicated a genomic organization of the four exons similar to that in the mouse (Fig. 2).

Construction of pXTRIL5 and Retroviral Expression of the Rat IL-5 Gene

A 4.1-kb AccI-HincII fragment containing some hundred base pairs of 5′ flanking regions, all rat IL-5 coding regions, and most of the 3′ untranslated region was inserted by several manipulations into the BglII cloning site of the retroviral vector pXT1,[21] resulting in pXTRIL5 (Fig. 2). This vector contains the neomycin gene as a selectable marker driven by the Moloney murine leukemia virus long terminal repeat (MoMuLV-LTR) and the rat IL-5 gene expressed from the internal herpes simplex virus–derived TK promoter. Psi_2-virus–producing cell lines were generated by transfection with pXTRIL5. A viral stock of the clone psi2-XTRIL5, which produces 1×10^4 neo^R colony forming units (CFU)/ml, was used to infect the two cell lines B13[22] and T88M,[23] whose growth is dependent on exogenous IL-5. The infected cells were first selected for neo^R and subsequently for IL-5 expression by omitting IL-5. From the cloning efficiency in the presence or absence of exogenous IL-5 we estimate that about 10% of the neo^R B13 or T88M cells were capable of growing factor independently. Since we could not detect spontaneous factor-independently growing cells, we concluded that the majority of IL-5–independent B13 or T88M cells were infected by the retrovirus pXTRIL5, leading to the expression of the rat IL-5 gene. To test this assumption, culture supernatants from five B13–derived (BR5) and eight T88M–derived (TR5) clones were assayed for IL-5 activity by a [^{3}H]-thymidine incorporation assay using T88M cells. The results for two BR5 and two TR5 clones are shown in Fig. 3 and demonstrate the presence of rat IL-5 acting across the species barrier on mouse cells. The proliferation of the indicator cells was IL-5 dependent because it could be inhibited (up to 70%) by the anti–IL5 receptor monoclonal antibody (mAb) R52.120,[22] but not by the isotype-matched anti–IL-4 mAb 11B11. In a control experiment, the same amount of 11B11 blocked the IL-4–dependent proliferation of CT.4S cells about 50%, whereas R52-120 had no effect, excluding a direct cytotoxic effect of R52-120. All clones tested expressed IL-5 activity. The bulk culture of BR5 cells secreted 0.3 U of IL-5 per milliliter and of TR5 cells 148 U of IL-5 per milliliter. Consistently, TR5 clones produced considerably more IL-5 than the BR5 clones. The psi2-XTRIL5 cells produced 2 U of IL-5 per milliliter, which should result from the expression of the genomic rat IL-5 gene (not shown).

Analysis of the Proviral Genome and Rescue of Retroviral IL-5 Sequences from BR5 and TR5 Cells

A Southern blot analysis was performed using the IL-5 probe, in order to examine the splicing of the rat IL-5 introns during the retroviral gene transfer. Upon EcoRI digestion a new 4.3-kb band which has the expected size of the retroviral "genomic" IL-5 fragment appears in psi2-XTRIL5 in addition to the endogenous 9.4- and 4-kb bands (Figs. 2, 4a). Other bands in psi_2 and psi2-XTRIL5 most likely result from cross-hybridization of pBR322 sequences in the probe with helper virus plasmid DNA in psi_2. In all XTRIL5-infected cells (four TR5, two BR5 clones) a new, lower molecular weight EcoRI band appears in addition to the presumably not rearranged endogenous bands. The 1.9-kb EcoRI fragment in TR5-1 to TR5-4 corresponds to the expected size of a retroviral "cDNA" IL-5 fragment whose introns were spliced precisely in the virus-producing cell line, although the band in TR5-2 seemed slightly larger (see below). In BR5-2 the

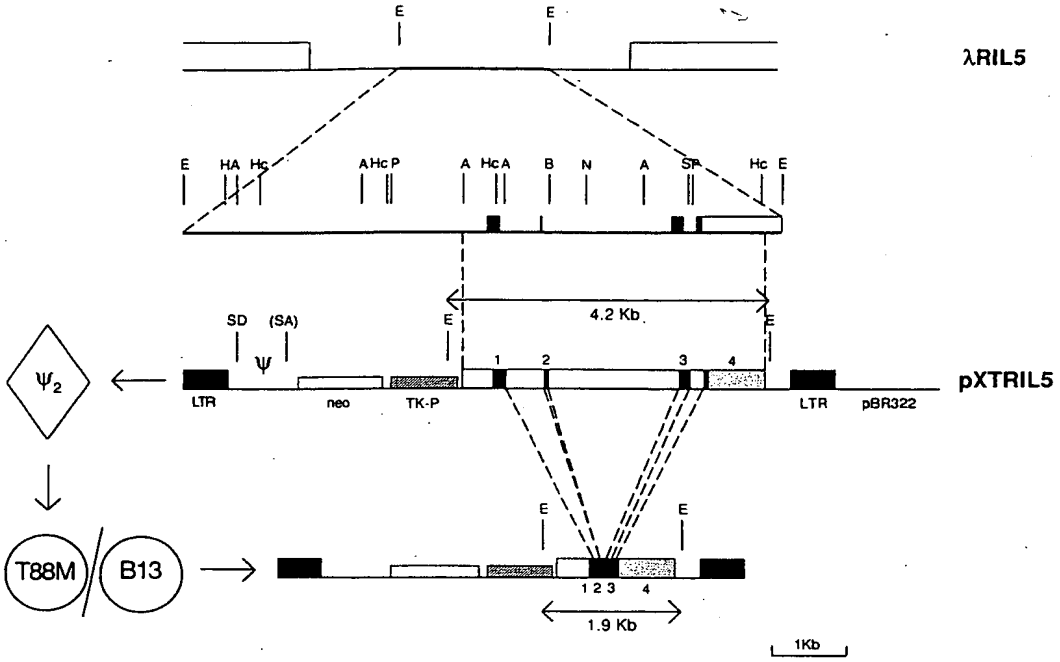

Figure 2. Schematic diagram showing the generation of a rat IL-5 "cDNA" by retroviral passage.

From top to bottom: lambda phage containing the genomic rat IL-5 gene and (below) a detailed restriction map of the 8-kb EcoRI fragment harboring the coding region (black boxes) and most of the 3′ untranslated region (open box). A 4.1-kb AccI-HincII fragment was cloned into the BglII site of pXT1 as described in Materials and Methods, giving rise to plasmid pXTRIL5. Only those EcoRI restriction sites are shown that are indicative of a rearranged IL-5 gene. A virus producing fibroblast cell line psi$_2$ was generated by transfection with pXTRIL5 and the virus was used to infect cell lines T88M and B13 from which IL-5 "cDNA" versions could be rescued. E, EcoRI; H, HindIII; A, AccI; Hc, HincII; P, PstI; B, BamHI; N, NcoI; S, SmaI; LTR, long terminal repeat; neo, neomycin; TK-P, thymidine kinase promoter; SD, splice donor site; (SA), cryptic splice acceptor site.

retroviral IL-5 band is 2.4 kb, suggesting different processing of the XTRIL5 mRNA. BR5-1 reveals additional 1.9- and 2.4-kb bands, indicating either that two viral integrations occurred or, what is more likely considering the reduced hybridization intensities of the additional bands, that BR5-1 is a mixture of two clones. In order to be sure that a functionally spliced IL-5 gene would be isolated from the genome of the infected cells, we wanted to confirm that only a single virus had been integrated. A Southern blot analysis with BclI-digested DNA shows, in comparison to T88M, one additional IL-5 band for the four TR5 clones, which is consistent with a single proviral integration, since BclI cuts only once within the proviral genome (Fig. 4c).

Next, fragments containing IL-5 sequences were rescued from XTRIL5 infected cells by PCR using primers specific for the TK promoter and MoMuLV sequences, which are located 5′ and 3′ of the BglII cloning site in pXT1, respectively. In addition, the primers contain cloning sites for either EcoRI or BamHI. The amplification products obtained from DNA of TR5-1 and BR5-2 were analyzed by a Southern blot using the IL-5 probe. After a 15 min exposure, bands appear corresponding in size to the retroviral

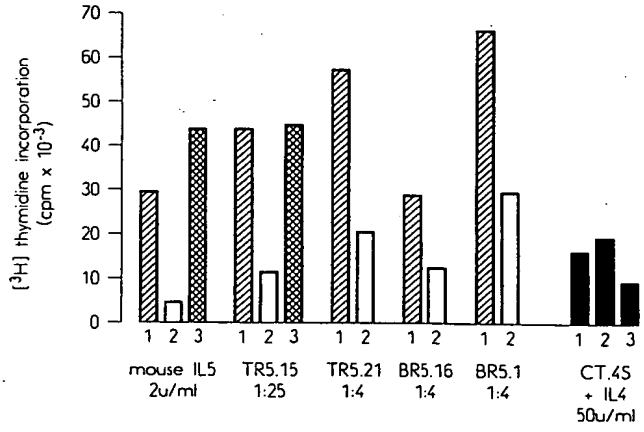

Figure 3. Cell proliferation assay measured by [^{3}H]-thymidine uptake using the IL-5 indicator cell line T88M in the presence of mouse IL-5 or diluted supernatants of cell lines as indicated.

(1) IL-5–dependent cell proliferation; (2) in the presence of anti–IL-5R mAb R52.120 (1:500 diluted ascites); (3) in the presence of anti–IL-4 mAb 11B11 (1:500 diluted ascites). The black bars show the IL-4–dependent proliferation of cell line CT.4S in the presence or absence of antibodies.

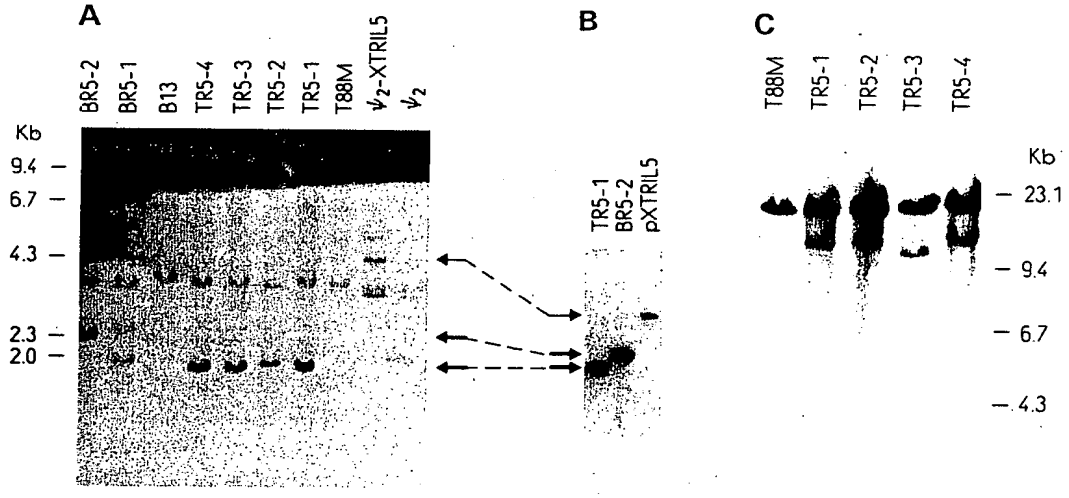

Figure 4. Analysis and rescue of the rat IL-5 "cDNA" generated by retroviral passage.

(A) EcoRI-digested DNA of indicated cell lines were hybridized in a Southern blot with the IL-5 probe. For the interpretation of altered IL-5 bands in TR5 and BR5 clones in comparison to psi_2-XTRIL5 see also Fig. 2. (B) PCR analysis of XTRIL5-infected T88M (TR5-1) and B13 cells (BR5-2). Amplification products from 50 ng of genomic and 80 pg of plasmid input DNA were electrophoresed on an agarose minigel after 26 cycles of amplification using primers specific for the TK promoter and MoMuLV sequences and subjected to Southern blot analysis using the IL-5 probe. Exposure time was 15 min. The size of the PCR products is 0.1 kb smaller than the corresponding EcoRI bands in (A) as indicated by the connecting arrows. (C) BclI-digested DNAs of T88M and TR5 cells were analyzed in a Southern blot using the IL-5 probe. The probe in A, B, and C was the 450-bp SacI-AccI mouse IL-5 cDNA fragment of pSP6K-mTRF23, and the size marker was lambda HindIII-digested DNA.

IL-5 fragments of the respective parental lines (Fig. 4b). Subsequently, the PCR-amplified rat IL-5 genes of five cell lines (TR5-1, TR5-2, TR5-3, BR5-2, BR5-3) were cloned into suitable vectors.

Characterization of the Rat IL-5 "cDNA"

The five rat IL-5 inserts were characterized by restriction analysis and partial sequence analysis. Clone TR5-1, containing the smallest proviral IL-5 fragment, was sequenced completely by the sequencing strategy outlined in Fig. 5. The sequence contains an open reading frame of 132 amino acids, which are 92% homologous to the mouse IL-5, 500 bp of 5′ flanking region, and 0.7 kb of 3′ untranslated region (Fig. 6). In addition, the sequence in Fig. 6 extends 0.3 kb in the 3′ direction up to the EcoRI site (see Fig. 2) and ends at a position which is homologous to a region 20 bp 5′ of the polyadenylation signal sequence in the mouse IL-5 gene. Sequencing of parts of other rescued "cDNA's" and genomic regions confirmed the sequence and demonstrated the presence of three introns flanked by splice donor and acceptor sites. We also noted a C-to-T mutation in the second position of amino acid 126 in TR5-1, leading to a change from threonine to isoleucine and a G-to-A mutation at position 1011 (see Fig. 6). The composite analysis of the five clones reveals three types of rat IL-5 sequences that have been rescued from the XTRIL5 infected cells (Fig. 5). TR5-1 and TR5-3 contain the expected, precisely spliced cDNA without deletions. Clone BR5-2 has retained the first intron. Similarly, TR5-2 and BR5-3 still possess the first intron but, in addition, have deleted most of the 5′ flanking region. In BR5-3 the deletion is 408 bp in length from nucleotides 60 to 467 (see Fig. 6). No adjacent splicing recognition sequences could be detected.

Identification of a Retrotransposon Inserted into the First Intron of the Mouse IL-5 Gene After Divergence from the Rat

Upon comparison of the overall genomic structure of human, rat, and mouse IL-5 genes and by sequencing of the rat IL-5 intron 1 region we noticed a gap of 303 bp within the rat sequence when compared to the mouse (Fig. 7). The insertion in mouse IL-5 intron 1 is flanked at either side by regions highly homologous between mouse and rat IL-5 genes and carries such features characteristic of retrotransposons as a 12-bp direct repeat most likely resulting from a target site duplication and a poly(A) tract at one end. The size of the first intron of the IL-5 gene in a number of laboratory and wild mice was analyzed in order to determine the time of the retrotransposon integration. To accomplish this, their genomic DNA was amplified

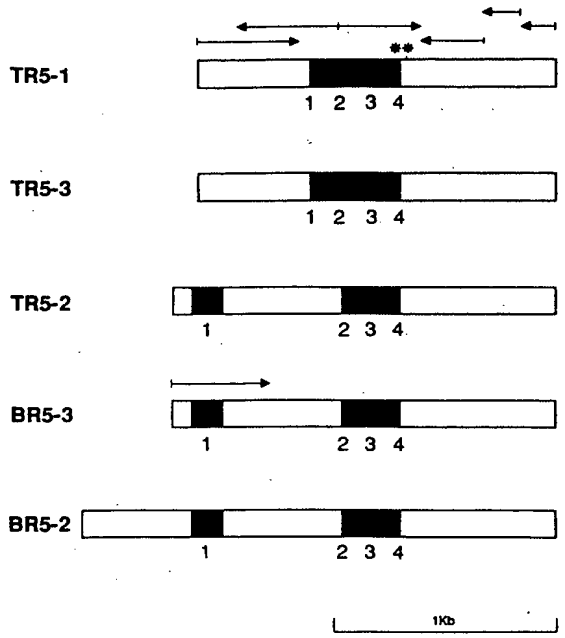

Figure 5. Schematic illustration showing the rat IL-5 regions rescued by PCR from five XTRIL5 infected cell lines.

Numbers refer to exons (black boxes). 5′ flanking and intron regions are shown as white and 3′ untranslated region as dotted boxes. Arrows indicate the sequencing strategy. The asterisks indicate mutations identified in TR5-1.

by PCR using primers specific for the first and the second exon of mouse and rat IL-5 genes (Fig. 8, top). The results show amplification products of about 950 bp for all the genomic DNA samples with the exception of those of the mouse *Mus coelomys pahari* and the rats. This fragment is indicative of the presence of the retrotransposon. The 650-bp fragment obtained with rat DNA shows the absence of the retrotransposon. The 1400-bp fragment amplified with *M.c. pahari* DNA indicates that either additional or different mutation events have occurred in the IL-5 intron 1. To confirm that specific fragments had been amplified, the agarose gel was subjected to Southern blot analysis using a rat intron 1–specific probe, demonstrating the expected hybridization (Fig. 8, bottom).

DISCUSSION

Our results demonstrate the useful combination of retroviral gene transfer and PCR for the characterization of genomic and cDNA regions and the parallel expression of genes previously analyzed in other species. This approach relies on the fact that genomic libraries from a large number of different species are available, that a probe from a related species exists to isolate the genomic region, and that intron sequences are removed during the retroviral life cycle. Since the retroviral vector sequences are known, the isolation of the "cDNA" is considerably facilitated by PCR, which furthermore restricts the generally time-consuming DNA sequence analysis to the informative exon regions. Following this experimental outline, we describe the isolation of the rat IL-5 gene, the generation and expression of its cDNA by retroviral gene transfer, the rescue of the cDNA by PCR, and the determination of its sequence. Target cells for the retroviral infection were IL-5–dependently growing cell lines, B13 and T88M. This made possible the selection of cells that had acquired a virus containing a functional IL-5 gene. In fact, all clones tested produced IL-5 between 2 and $>1{,}000$ U. On the other hand, the number of virus particles transfering an aberrantly spliced IL-5 gene seemed to be low, since the frequency of rat IL-5–independent $G418^R$ cells was similar to that of cells infected with a retrovirus that contained a mouse IL-5 cDNA region (not shown).

The rescue of the five rearranged proviral rat IL-5 sequences shows two cases in which all three introns were spliced. In three other cases the first intron was retained but the second and third introns were deleted. Two of these suffer from a deletion of the rat IL-5 "promoter" region. Despite the existence of these differently reassembled functional IL-5 regions, we could not detect a correlation between any unusual processing and level of IL-5 activity. Therefore, selection presumably does not account for the identification of incomplete splicing of the first intron in three out of five cases. It could, rather, reflect a certain order during processing of the primary IL-5 transcript, or, alternatively, the preferential packaging of certain retroviral transcripts. To address this question in more detail, we are currently analyzing—again with the help of PCR—the content of retrovirus-specific transcripts which are generated in the virus-producing cell line, which are encapsicated and released into the culture medium, which are able to infect the target cell, and which contain a functional IL-5 gene.

A disadvantage of the approach used is the high mutation rate arising from the use of the reverse transcriptase or, more likely, the Taq polymerase. Indeed, two mutations were detected in the sequence of TR5-1, one of which led to an amino acid exchange in position 126. The mutation rate of 10^{-3} ($2/\approx 2000$ bp) is compatible with the error rate of the Taq polymerase and may be overcome by direct sequencing of the amplified products.[24]

The rat IL-5 gene was characterized because we recently noticed an unusual high divergence between mouse and rat IL-4 genes (57% amino acid homology).[7] Both lymphokines, IL-4 and IL-5, are simultaneously synthesized by T lymphocytes preferentially of the TH2 type.[8] Both stimulate growth and affect differentiation of B cells.[11] In mouse and human the

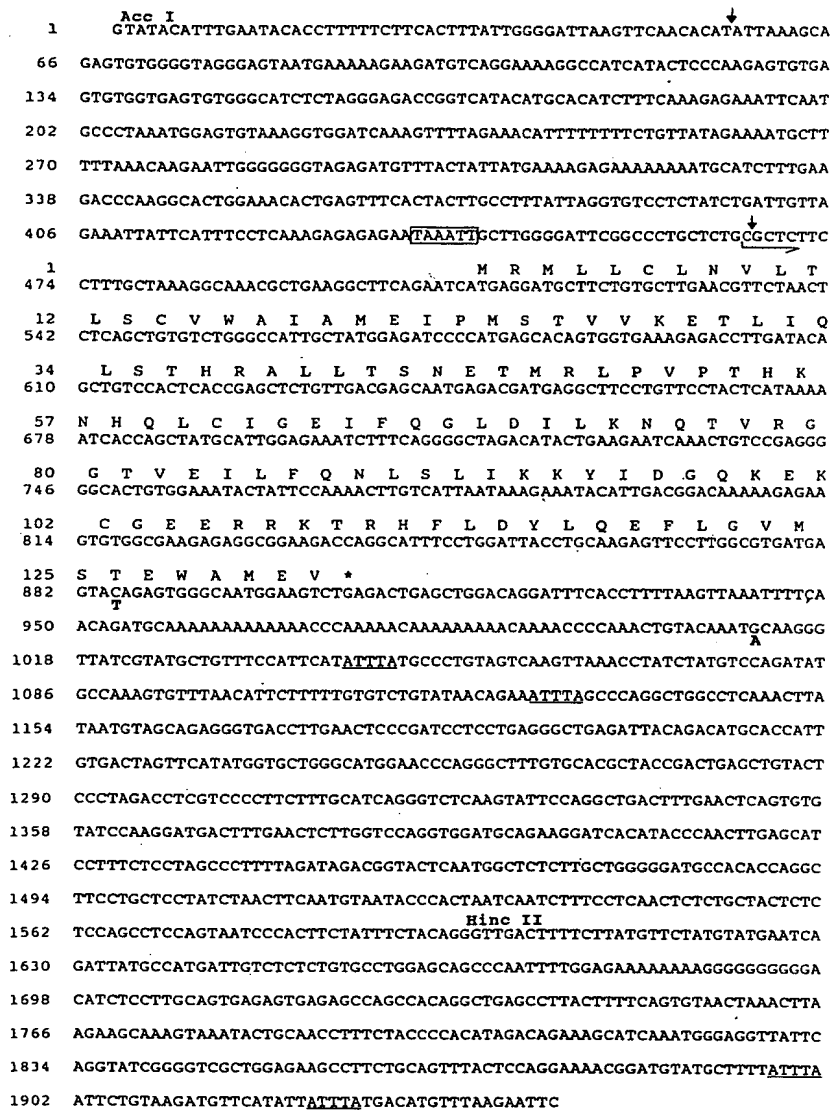

Figure 6. Nucleotide seqeunce of the rat IL-5 "cDNA" isolated from the cell line TR5-1.

Coding regions have been deduced by comparison to the mouse IL-5 gene and are indicated by amino acid translation above the sequence. The sequence 3′ of the HincII site at position 1593-1603 was derived from the rat IL-5 genomic fragment. The position for consensus sequences of a TATA box is marked by a closed box, the transcription initiation site deduced from the mouse IL-5 gene is indicated by a horizontal arrow, and signal sequences for potential mRNA destabilization signals are underlined. The mutations in TR5-1 are shown below the sequence. Vertical arrows indicate the endpoints of the deletion in the promoter region of BR5-3. These sequence data have been submitted to the EMBL/Gene Bank Data Libraries under the accession numbers X54419.

IL-4 and IL-5 genes are located close to each other on the same chromosome.[9,10] In contrast to the IL-4 genes, the comparison of rat and mouse IL-5 genes shows their strong evolutionary conservation: both consist of a very similar exon/intron structure and size and their exon regions are 94 and 92% homologous to each other at the nucleotide and the amino acid level, respectively (Fig. 9). Consistently with this is the fact that the rat IL-5 acts on mouse cells, whereas the IL-4 genes, which show low homology between mouse and rat, do not act across the species barrier. Rat and human IL-5 proteins are 68% homologous. The 500-bp presumed promotor regions are 83% homologous between mouse and rat. Twelve base pairs 5′ of a potential TATA box the rat IL-5 gene contains a consensus region which is found within the promotor region of the hematopoetic growth-factors, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5.[6] In the 3′ untranslated part, the rat IL-5 sequence contains four ATTTA motifs which are highly conserved among cytokine genes and which may be involved in rapid degradation of the IL-5 mRNA.[25]

Upon comparison of mouse and rat IL-5 genes we noticed the insertion of a 303-bp fragment into the mouse intron 1 region. The presence of a target site duplication and a poly(A) tract support the assumption that the fragment is integrated by retrotransposition. The retrotransposon is not homologous to any known sequence, except for a 110-bp region consisting of C's and T's exclusively. Although abundantly present in the genome, this repetitive sequence has not yet been associated with retrotransposition. PCR amplification and size determination of the IL-5 intron 1 region in a number of different murine species indicated that the retrotransposon seems to be present in all species but

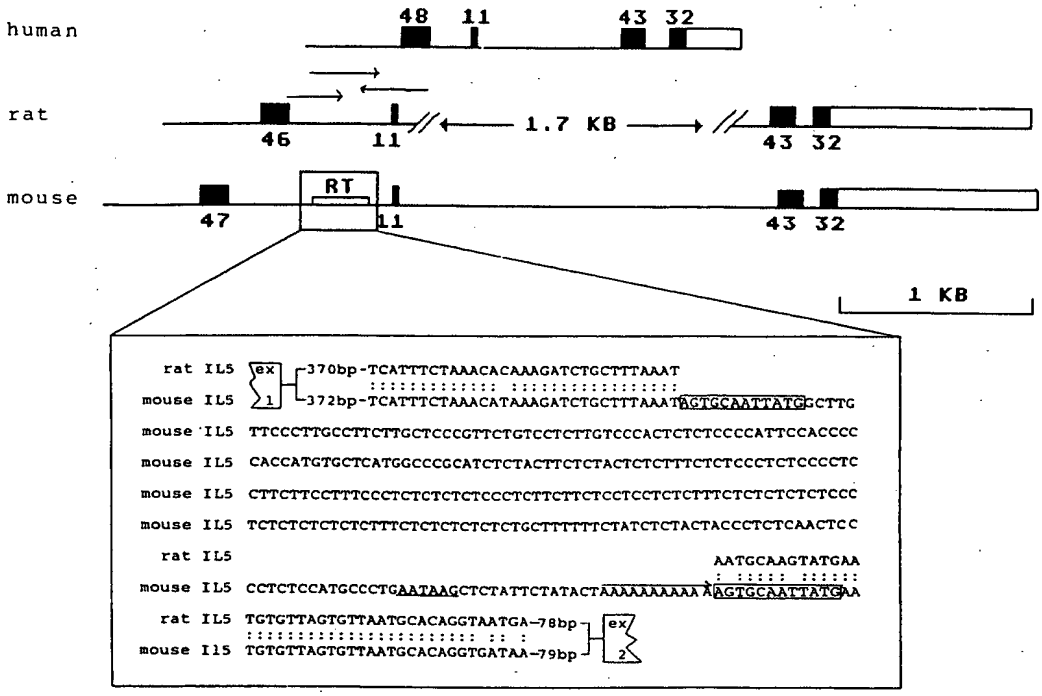

Figure 7. Comparison of human, rat, and mouse IL-5 genes.

(Top) Comparison of genomic regions of human, rat, and mouse IL-5 genes. Coding regions are marked by black, 3′ untranslated regions by open boxes. The numbers of amino acids which encode the individual exons are indicated. The integration of a retrotransposon (RT) in mouse intron 1 is marked by a small open box. (Bottom) Comparison of mouse and rat IL-5 intron 1 sequences close to the region which flanks the retrotransposon in the mouse. The direct repeats are boxed, the potential polyadenylation signal is underlined. The poly(A) stretch is marked by an arrow. The distances to exon 1 and 2 in both mouse and rat genes are indicated. The sequence of rat IL-5 intron 1 has been submitted to the EMBL/Gene Bank Data libraries under the accession number X52845.

the rat. Therefore, the retrotransposon should have integrated into the IL-5 intron 1 after mouse and rat diverged from each other but before most murine species separated from each other.

MATERIALS AND METHODS

Construction and Screening of a Rat Genomic Library

Twelve- to twenty-kB enriched fragments of partially Sau3A-digested Lewis rat liver DNA were ligated into BamHI-digested EMBL3 arms and packaged in vitro resulting in 1.5×10^6 recombinant phages. EMBL3 arms and Gigapack Gold packaging extracts were purchased from Stratagene, La Jolla, CA. Plaque hybridization to the 630-bp BamHI-AccI IL-5 fragment of plasmid pSP6K-mTRF23, lambda phage growth, plaque purification, lambda DNA extraction, restriction analysis, and subcloning of restriction fragments were done according to standard procedures.[26]

Generation of the Rat IL-5 Recombinant Retrovirus

The retroviral vector pXTRIL5 was constructed by inserting a 4.1-kb AccI-HincII fragment containing the rat IL-5 gene into the plasmid pXT1 behind the thymidine kinase (TK) promoter.[21] First, a 0.6-kb AccI-HincII fragment containing 500 bp of the 5′ region and part of exon 1 was blunt-ended with Klenow polymerase, inserted into HincII-SmaI pretreated pBluescript plasmid, and excised as a BamHI-HincII fragment. Second, a 3.5-kb HincII fragment which contains parts of exon 1, exon 2, 3, and 4, and 700 bp of the 3′ untranslated region, but which has the last 300 bp and the polyadenylation signal deleted, was cloned into the HincII and SmaI sites of pBluescript and recovered by HincII and partial BamHI digestion. Finally, the 0.6-kb BamHI-HincII 5′ fragment, the 3.5-kb HincII-BamHI 3′ rat IL-5 fragment, and the BglII-digested and dephosphorylated pXT1 were ligated, resulting in pXTRIL5. The cell line psi_2[27] was transfected with pXTRIL5 using a mammalian transfection kit (Stratagene, La Jolla, CA) and selected in medium containing 1 mg/ml of G418, and clones were established producing recombinant, helper-free viruses. The virus titers were measured by determination of the number of G418 (1 mg/ml) resistant colonies (CFU/ml) after infection of cultures of 1×10^5 NIH 3T3 cells with serial dilutions of supernatants derived from 1×10^6 virus producing cells per milliliter cultured for 24 h.

Cell Lines

Psi_2[27] and NIH 3T3 cells were maintained in Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. Cell lines B13[22]

and T88M[23] were cultured in RPMI-1640 (Seromed) supplemented with 10% FCS, 50 μM 2-mercaptoethanol, and 20 U/ml of IL-5. Both cell lines seem to represent immature B cells, and their growth is dependent on exogenous IL-5. CT.4S is an IL-4–dependently growing derivative of CTLL[28] and has been cultured as described.[29]

Retroviral Infection

Cells were cultured for 12 to 16 h in the presence of culture supernatants derived from psi_2-XTRIL5 cells supplemented with 4 μg/ml polybrene and 20 U/ml of IL-5. Afterwards, the culture supernatant was substituted by fresh medium containing 1 mg/ml G418 to select for infected cells.

Southern Blot Analysis

High molecular weight liver DNA of a BALB/c, C3H, DBA/2, MOLF/EI (inbred *Mus musculus molossinus*), CAST/EI (*Mus musculus castaneus*), *M. musculus musculus, M. spretus, M. caroli, M. cookii, M. pyromys saxicola, M. coelomys pahari* mice, and Wistar and Lewis rats and DNA of cell lines, as indicated in the text, were extracted as described.[26,30] Southern blot analysis was performed as previously described.[7] Either the nick-translated 630-bp BamHI-AccI or the 450-bp SacI-AccI fragment of plasmid pSP6K-mTRF23[3] was used as probe.

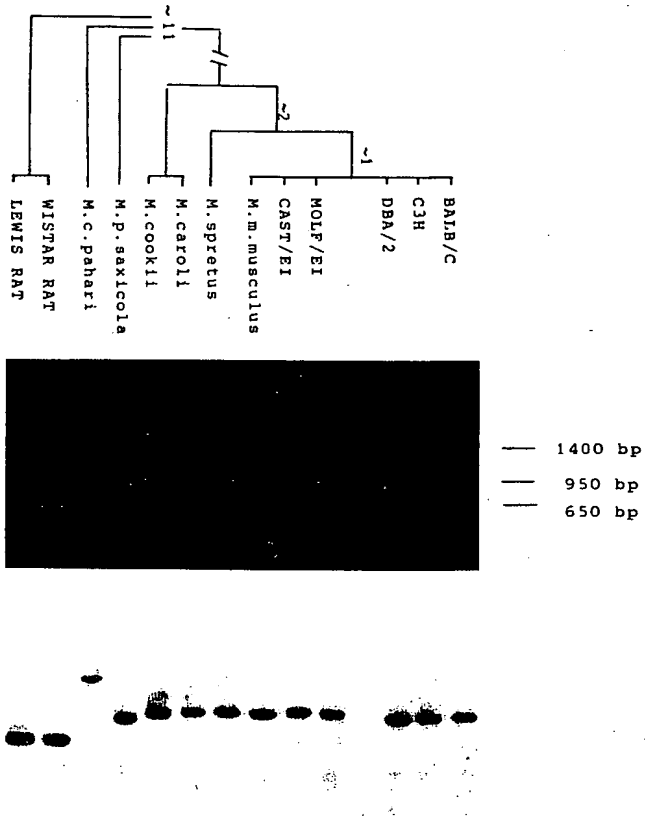

Figure 8. Analysis of sizes of IL-5 intron 1 regions from various species.

(Top) Analysis of the size of IL-5 intron 1 region of indicated animals by PCR using primers specific for exon 1 and exon 2. The tentative times of radiation are indicated. (Bottom) The same agarose gel was subjected to Southern blot analysis using a rat IL-5 intron 1–specific probe. The exposure time was 30 min.

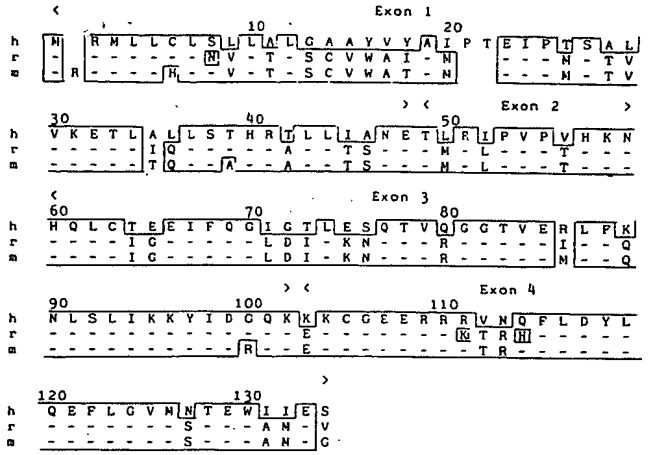

Figure 9. Comparison of human, rat, and mouse IL-5 amino acid sequences.

Identical residues are indicated by dashes and have been boxed. The exon regions are indicated above the sequences.

Polymerase Chain Reaction

The polymerase chain reaction was performed in a 100-μl volume containing 500 ng of genomic DNA, 0.1 μM of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.4 at RT), 1.5 mM $MgCl_2$, 100 μg/ml gelatin, and 200 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP). The template DNA was denatured for 5 min at 94°C, primer annealing was allowed to occur at 55°C for 1 min, 2 U of Taq polymerase (Amersham) were added, and the first primer extension proceeded at 72°C for 1½ min. Then, amplification occurred during 25 cycles of denaturing at 94°C (30 sec), annealing at 55°C (60 sec), and elongation at 72°C (90 sec). An aliquot of the amplified fragments was size-separated by agarose gel electrophoresis and specificity was controlled by Southern blot analysis using the IL-5 probe (see above). The following two pairs of primers have been used: (1) 5′-GGAATTCAACAGCGTGCCGCA-3′ and 5′-GGAATTCCCCTGGCGGACGGG-3′ and (2) 5′-GCGAATTCGAACACGCAGATGCAG-3′ and 5′-CGGATCCCCTGGCGGACGGGAAGT-3′. Each set contains a primer which is specific for a region 5′ of the BglII cloning site in the TK promoter.[31] The corresponding second primer is specific for Moloney murine leukemia virus (MoMuLV) sequences located 3′ of the BglII site in pXT1. In addition, the primers contain recognition sequences for EcoRI or BamHI. An aliquot of the amplified fragment was digested with EcoRI (first primer pairs) or EcoRI/BamHI (second primer pairs), cloned into M13 vectors, and subjected to DNA sequence analysis. For the amplification of the first intron of murine IL-5 genes the following primer pair was used: 5′-ACTCTCAGCTGTGTCTGGGCCA-3′ and 5′-GTAGGGACAGGAAGCCTCATCGT-3′. The polymerase chain reaction mix additionally contained 1 U of "perfect match" (Stratagene). The annealing temperature was 60°C.

DNA Sequence Analysis

Nucleotide sequence analysis was performed by the dideoxy chain termination method.[31] For the processing of DNA sequence data the DNASIS program (Pharmacia, Uppsala, Sweden) was used.

Assay for IL-5 Activity

The biological activity of IL-5 was measured by [^{3}H]-thymidine incorporation of T88M cells. Cells (5×10^3 cells/0.2 ml) cultured in microtiter plates were incubated for 24 h with serial dilutions of IL-5–containing supernatants. Cells were pulse-labeled with 0.5 μCi/well [^{3}H]-thymidine during another 12-h culture period, and DNA synthesis was determined by [^{3}H]-thymidine incorporation. One unit of IL-5 activity was defined as the amount of IL-5 required for half-maximal proliferation. In some experiments, cell proliferation was determined in the presence of the rat anti–mouse-IL5-receptor mAb R52.120 (IgG1/K)[22] or the rat anti–mouse-IL4 mAb 11B11 (IgG1/K).[32]

Acknowledgments

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REFERENCES

1. Takatsu K, Tominaga A, Harada N, Mita S, Matsumoto M, Takahashi T, Kikuchi Y, Yamaguchi N (1988) T cell-replacing factor (TRF)/interleukin 5 (IL5): molecular and functional properties. Immunol Rev 102:107-136.

2. Sideras P, Noma T, Honjo T (1988) Structure and function of interleukin 4 and 5. Immunol Rev 102:189-212.

3. Kinashi T, Harada N, Severinson E, Tanabe T, Sideras P, Konishi M, Azuma C, Tominaga A, Bergstedt-Lindqvist S, Takahashi M, Matsuda F, Yaoita Y, Takatsu K, Honjo T (1986) Cloning of complementary DNA encoding T-cell replacing factor and identity with B-cell growth factor II. Nature 324:70-73.

4. Yokota T, Coffman RL, Hagiwara H, Rennick DM, Takebe Y, Yokota K, Gemmel L, Shrader B, Yang G, Meyerson P, Luh J, Hoy P, Pene J, Briere F, Spits H, Banchereau J, de Vries J, Lee FD, Arai N, Arai K (1987) Isolation and characterization of lymphokine cDNA clones encoding mouse and human IgA-enhancing factor and eosinophil colony-stimulating factor activities: relationship to interleukin 5. Proc Natl Acad Sci USA 84:7388-7392.

5. Tanabe T, Konishi M, Mizuta T, Noma T, Honjo T (1987) Molecular cloning and structure of the human interleukin-5 gene. J Biol Chem 262:16580-16584.

6. Campbell HD, Sanderson CJ, Wang Y, Hort Y, Martinson ME, Tucker WQJ, Stellwagen A, Strath M, Young IG (1988) Isolation, structure and expression of cDNA and genomic clones for murine eosinophil differentiation factor. Eur J Biochem 174:345-352.

7. Richter G, Blankenstein T, Diamantstein T (1990) Evolutionary aspects, structure, and expression of the rat interleukin 4 gene. Cytokine 2:221-228.

8. Coffman RL, Seymour BW, Lebman DA, Hiraki DD, Christiansen JA, Shrader B, Cherwinski HM, Savelkoul HF, Finkelman FD, Bond MW, Mosman TR (1988). The role of helper T-cell products in mouse B-cell differentiation and isotype regulation. Immunol Rev 102:5-28.

9. van Leeuwen BH, Martinsom ME, Webb GC, Young IG (1989) Molecular organization of the cytokine gene cluster, involving the human IL-3, IL-4, IL-5, and GM-CSF genes, on chromosome 5. Blood 73:1142-1148.

10. Lee JS, Campbell HD, Kozak CA, Young IG (1989) The IL-4 and IL-5 genes are closely linked and are part of a cytokine gene cluster on mouse chromosome 11. Somatic Cell Mol Genet 15:143-152.

11. Swain SL, McKenzie DT, Dutton RW, Tonkonogy SL, English M (1988) The role of IL4 and IL5: characterisation of a distinct helper T cell subset that makes IL4 and IL5 (TH2) and requires priming before induction of lymphokine secretion. Immunol Rev 102:77-105.

12. Shimotohno K, Temin HM (1982) Loss of intervening sequences in genomic mouse α-globin DNA inserted in an infectious retrovirus vector. Nature 299:265-268.

13. Cepko CL, Roberts BE, Mulligan RC (1988) Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell 37:1053-1062.

14. Weis JH, Reiss CS, Mulligan RC, Burakoff SJ, Seidman JG (1985) H-2L^d antigen encoded by a recombinant retrovirus genome is expressed on the surface of infected cells. Mol Cell Biol 5:1379-1384.

15. Brown AMC, Wildin RS, Prendergast TJ, Varmus HE (1986) A retrovirus vector expressing the putative mammary oncogene int-1 causes partial transformation of a mammary epithelial cell line. Cell 46:1001-1009.

16. Rijsewijk FAM, van Lohuizen M, van Ooyen A, Nusse R (1986) Construction of a retroviral cDNA version of the int-1 mammary oncogene and its expression in vitro. Nucleic Acids Res 14:693-702.

17. Auffray C, Lillie JW, Korman AJ, Boss JM, Fréchin N, Guillemot F, Cooper J, Mulligan RC, Strominger JL (1987) Structure and expression of HLA-DQ$_\alpha$ gene and functional splicing of the HLA-DX$_\alpha$ gene using a retroviral vector. Immunogenetics 26:63-73.

18. Schwarzbauer JE, Mulligan RC, Hynes RO (1987) Efficient and stable expression of recombinant fibronectin polypeptides. Proc Natl Acad Sci USA 84:754-758.

19. Perkins AS, Kirschmeier PT, Weinstein B (1989) Transduction of the human insulin gene via retroviral vectors fails to yield spliced transcripts. DNA 8:59-68.

20. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350-1354.

21. Boulter CA, Wagner EF (1987) A universal retroviral vector for efficient constitutive expression of exogenous genes. Nucleic Acids Res 15:7194.

22. Rolink AG, Melchers F, Palacios R (1989) Monoclonal antibodies reactive with the mouse interleukin 5 receptor. J Exp Med 169:1693-1701.

23. Mita S, Tominaga A, Hitoshi Y, Sakamoto K, Honjo T, Akagi M, Kikuchi Y, Yamaguchi N, Takatsu K (1989) Characterization of high-affinity receptors for interleukin 5 on interleukin 5-dependent cell lines. Proc Natl Acad Sci USA 86:2311-2315.

24. Gyllenstein UB, Erlich HA (1988) Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc Natl Acad Sci USA 85:7652-7656.

25. Malter J (1989) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Science 246:664-666.

26. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor University Press, Cold Spring Harbor, NY.

27. Mann R, Mulligan RC, Baltimore D (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell 33:153-159.

28. Hu-Li J, Ohara J, Watson C, Tsang W, Paul WE (1989) Derivation of a T cell line that is highly responsive to IL4 and IL2 (CT.4R) and of an IL2 hyporesponsive mutant of that line (CT.4S). J Immunol 142:800-807.

29. Blankenstein T, Li W, Müller W, Diamantstein T (1990) Retroviral interleukin 4 gene transfer into an interleukin 4-dependent cell line results in autocrine growth but not in tumorigenicity. Eur J Immunol 20:935-938.

30. Blankenstein T, Bonhomme F, Krawinkel U (1987) Evolution of pseudogenes in the immunoglobulin V_H-gene family of the mouse. Immunogenetics 26:237-248.

31. Garapin AC, Colbère-Garapin F, Cohen-Solal M, Horodniceanu F, Kourilsky P (1981) Expression of herpes simplex virus type I thymidine kinase gene in Escherichia coli. Proc Natl Acad Sci USA 78:815-819.

32. Sanger F, Coulson AR, Barrell BG, Smith AJH, Roe BA (1980) Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J Mol Biol 143:161-178.

33. Ohara J, Paul WE (1985) Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. Nature 315:333-336.

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Structure of the Rat Interleukin 6 Gene and Its Expression in Macrophage-derived Cells*

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Rat peritoneal exudate cells produce two interleukin 6 (IL6) messenger RNA species, a major 1200 nucleotide and a 5-fold less abundant, 2400-nucleotide species. A cDNA clone representing the major species was isolated, and sequenced. The 1055-base pair insert covered the 3′-nontranslated region, the 211 triplet coding region and most of the 5′-nontranslated region. The derived rat IL6 amino acid sequence was 93 and 58% identical, respectively, with mature murine and human IL6. Rat IL6 lacks *N*-glycosylation sites but contains a fifth cysteinyl residue in addition to the 4 residues shared in conserved positions with murine and human IL6. Stable murine L cell and human HeLa-derived cell lines were established by cotransfection with rat IL6 cDNA and a selectable neomycin resistance marker. These lines secrete 9-fold increased amounts of functional IL6 over their respective parental cells. A stable rat macrophage-derived cell line, RM-SV1, was established by transformation with simian virus 40. IL6 and Il1 mRNA levels are inducible 20- and 3.5-fold, respectively, in this line by treatment with lipopolysaccharide with kinetics characteristic of macrophages.

A set of three overlapping genomic DNA clones was isolated and a 10-kilobase DNA segment was sequenced containing the rat IL6 gene plus 2.9 kilobases of 5′-flanking and 1.3 kilobases of 3′-flanking sequences. The two transcription start sites used in RM-SV1 cells were mapped within 5 base pairs of each other. The exon/intron boundaries are conserved with the murine and human IL6 genes. The two IL6 mRNA species are generated by alternative polyadenylation at sites separated by a distance of 1.2 kilobases. The intervening region contains a repetitive element 72–80% identical with the rat and murine consensus L1 family sequences.

Interleukin 6 (IL6)[1] is a multifunctional cytokine with both differentiation and growth-promoting effects for a variety of target cell types. It is a B-cell stimulatory factor inducing terminal differentiation and high level antibody production in B lymphocytes (IL6/BSF-2, 1, 2), and it acts as a hybridoma/plasmacytoma growth factor (3, 4). It promotes growth of B-myeloma cells as well as T-lymphocyte growth and differentiation (1, 5, 6), but it inhibits the growth of primary rat hepatocytes in culture (7). The hormone stimulates the growth of hemopoietic stem cells, and in synergism with interleukin 3 (IL3) it supports the proliferation of cultured multipotential hemopoietic progenitor cells (8, 9). IL6 is a mediator of inflammation and a major alarm hormone signaling tissue damage and infection to the body's host defense system and in particular to the liver. Its ability to induce the synthesis of acute phase plasma proteins in hepatocytes is referred to as its hepatocyte stimulatory function (IL6/HSF, 10; reviewed in 1, 11). This cytokine also causes fever in rabbits and humans and is therefore suspected to have a direct effect on cells of the nervous system (12).

IL6 is produced by many different cell types including fibroblasts, macrophages/monocytes, T and B lymphocytes, endothelial cells, synovial cells, keratinocytes, and by a number of tumor-derived cell lines (1, 11, 13–16) including several rat and human hepatoma cell lines (17, 18). Although the IL6 gene is expressed at low levels in a large number of cell types (19), the cell types listed here and various tumor cells are the most abundant producers of IL6 in mammals.

Expression of the IL6 gene can be induced by a number of different signals, including cytokines (interleukin 1, IL1; tumor necrosis factor α, platelet-derived growth factor, interferon β_1, and epidermal growth factor), serum, double-stranded polyribonucleotides, cycloheximide, phorbolesters, agents that increase intracellular cyclic AMP, viral infections, and bacterial endotoxins (1, 11, 20–26). Induction of the IL6 gene is a very early event in the inflammatory response that leads to important secondary effects on the homeostatic defense system (52). In addition, it has been hypothesized that activation of the IL6 gene may not only be a secondary consequence of oncogenic growth transformation of cells but may itself be part of the initial events leading to oncogenic transformation (1, 11, 17). Therefore, it is important to understand the molecular mechanisms causing the activation of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M26744 and M26745.

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[1] The abbreviations used are: IL1, IL6, interleukin 1, interleukin 6; HSF, hepatocyte stimulating factor; BSF-2, B-cell stimulatory factor (According to a nomenclature convention reached at a meeting on IL6 of the New York Academy of Sciences in December 1988, IL6/HSF refers to the HSF function of IL6, IL6/BSF-2 to the BSF-2 function, IL6/HGF to the HGF function, etc.); LPS, lipopolysaccharide; SDS, sodium dodecyl sulfate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PECs, peritoneal exudate cells; SSC, standard sodium citrate; bp, base pairs; kb, kilobases.

this gene. As a first step in this direction, we have isolated and sequenced rat IL6 cDNA and genomic DNA clones.

Here we report the gene and cDNA sequences and the cDNA-derived amino acid sequence of rat IL6 and compare it with its murine and human counterparts. We describe the establishment of a rat macrophage-derived cell line that was used as a convenient source of mRNA and as a tool for studies of the expression of this gene in macrophage-like cells. We also report the establishment of murine L cell and human HeLa-derived cell lines that express and secrete functionally active recombinant rat IL6. The exon/intron block structure of the gene has been determined, and we report the presence of two IL6 mRNA species derived from the single copy rat IL6 gene by alternative polyadenylation.

EXPERIMENTAL PROCEDURES

Animals and Materials—Male Fisher 344 rats (Simonsen Laboratories, Gilroy, CA) were used for the preparation of primary peritoneal exudate cell cultures and RNA preparation. Laboratory chemicals and standard enzymes for molecular biology were from J. T. Baker Chemical Co., Mallinckrodt, Fisher, Sigma, New England Biolabs, Bethesda Research Laboratories, Stratagene, United States Biochemicals, and Boehringer Mannheim as described (27). Fetal calf serum and tissue culture supplies were from GIBCO and Flow Laboratories, and radionucleotides from Amersham Corp. and ICN. Lipopolysaccharide was from Sigma.

Peritoneal Exudate Cell Cultures and RNA Preparations—After euthanasia with CO_2 gas, the peritoneal lavage was performed by two consecutive intraperitoneal injections of 35 ml of serum free, ice-cold Dulbecco's modified minimal essential medium (Scripps Media Production) into rats. The lavage fluid was collected, and the peritoneal cells were recovered by low speed centrifugation. The cells were resuspended in serum-free medium, washed by another cycle of centrifugation and resuspension, then plated in tissue culture Petri dishes and allowed to attach for 4 h at 37 °C. Nonadherent cells were removed by two cycles of washing with phosphate-buffered saline, and the cells were supplied with Dulbecco's modified minimal essential medium containing 10% fetal bovine serum and kept at 37 °C for 20 h. Supernatants were then collected and assayed for IL6/HSF activity, and RNA was prepared by lysis of the cells in guanidinium thiocyanate and high speed sedimentation of the RNA through a cesium chloride cushion (28). Northern blot analysis was performed in agarose formaldehyde gels as previously described (29, 30). Northern blots were hybridized in a solution containing 50% formamide, 20 mM PIPES, pH 6.5, 0.8 M sodium chloride, 1 mM EDTA, 300 μg/ml of denatured salmon sperm DNA, and 0.5% sodium dodecyl sulfate (SDS) for 24 h at 42 °C. They were washed two times for 30 min in 500 ml of wash solution at 42 °C. The first wash solution was 0.1% SDS containing 2 × standard sodium citrate (SSC), the second wash solution contained 0.1% SDS in 1 × SSC. SSC is 0.15 M sodium chloride, containing 0.015 M sodium citrate.

Preparation and Screening of cDNA Libraries—The first cDNA library was produced using total RNA prepared as described above from adherent primary rat peritoneal exudate cells in the vector pGEXII, a commercial derivative (Genecell, Inc., San Diego) of the Okayama and Berg vector pcDV1 (31). Approximately 60,000 colonies of a cDNA library consisting of 40,000 independent clones were hybridized with a murine IL6 cDNA probe (32). The second cDNA library was produced from polyadenylated mRNA prepared from the rat macrophage cell line RM-SV1 (see "Results") in the commercial vector λZAPII (Stratagene, Inc., San Diego). The second library was screened with the rat IL6 cDNA clone pIL6C.91 isolated from the first library and with a 1.22-kb *Sac*I/*Bam*HI genomic DNA fragment from the 3′ end of the gene (see "Results").

DNA Sequencing and Computer-assisted Data Analysis—Random subfragments of cDNA and genomic DNA clones were generated by sonication and were subcloned into M13 phage vectors. DNA sequencing with the dideoxynucleotide technique and sequenase enzyme (USB) followed standard procedures (34). Sequencing data were collected and analyzed using Staden's database system (35) and the University of Wisconsin Genetics Computer Group programs (36) on a Digital VAX 11/750 computer. The genomic DNA sequence of 10,067 nucleotides was derived from a total database of 63,252 nucleotides. The average nucleotide of the sequence was covered by 6.3 independent sequence gel readings, and 100% of the sequence was covered on both strands. The sequence of the cDNA clone representing the major 1.2-kb mRNA species with an insert of 1,055 base pairs (bp) was derived from a total database of 2,514 characters. Only 85% were covered on both strands, but the sequence was matched completely by the previously established genomic DNA sequence. The cDNA representing the 2.4-kb mRNA species had an insert length of 1,909 nucleotides and was covered by a total database of 6,343 nucleotides. Its sequence was matched completely by the genomic DNA sequence and by the sequence of the cDNA clone representing the 1.2-kb mRNA species. For some cDNA clones the terminal sequences of the inserts were determined using the double-stranded plasmid DNA sequencing method (37).

Biological Assay for IL6/HSF Function—A novel indicator cell line for IL6/HSF function and a novel assay were established (17).[2] The indicator line FaO/HB3 is a derivative of the well-characterized rat hepatoma cell line FaO, which was previously shown to respond to treatment with IL6/HSF by increased transcription of the α_2-macroglobulin gene and other characteristic acute phase genes (17, 38). The FaO/HB3 line was established by cotransfection of FaO cells with a plasmid carrying the 5′-flanking control region of the rat α_2-macroglobulin gene (17, 39, 40) directing the expression of the firefly luciferase gene (41) and a neomycin (G418) resistance marker. FaO/HB3 cells respond to treatment with IL6/HSF by production of luciferase activity. For the assay, FaO/HB3 cells were grown to confluence, then split 1:1 and allowed to grow overnight (12 h) in presence of 10^{-7} M dexamethasone. The cells were then treated for 4–5 h with various test supernatants of cultured cells or with known concentrations of purified recombinant human IL6 produced in *Escherichia coli*. This material, with a specific activity of 5×10^6 IL6/BSF-2 units/mg, was obtained from Dr. Asagoe and colleagues, Tosoh Inc., Japan and Drs. Hirano and Kishimoto, Osaka, Japan. FaO/HB3 cell extracts were prepared according to a standard procedure[3] and used for luciferase assays in a luminometer (Monolight 2001; Analytical Bioluminescence, Inc., San Diego, CA) following the instructions of the supplier (41). The protein concentrations in the extracts were determined with a color assay (Bio-Rad), and the luciferase activities were normalized to constant amounts of extracts, usually to 200 μg of protein (see "Results," Table I). The luciferase activities were then converted to IL6/BSF-2 units using a calibration with known amounts of recombinant human IL6.

Screening Genomic Libraries and Characterization of Genomic DNA Clones—A rat genomic DNA library, constructed with *Eco*RI partial restriction fragments in the phage vector λ charon 4A (42) was screened with the rat IL6 cDNA clone pRIL6C.91. Screening and characterization of the isolated clones by restriction enzyme cleavage mapping were performed as described (39).

RNase Protection Assays and RNase H Experiments—To map the 5′ ends of IL6 mRNA by RNase protection, a 1.205-kb *Hin*dIII/*Bst*XI genomic DNA fragment containing exon 1 (see "Results," Fig. 9) was blunt-ended with T4 DNA polymerase and subcloned into the *Sma*I site in the polylinker of the Bluescript® KS vector (Stratagene). This construct linearized with *Bam*HI was used to generate an internally radiolabeled RNA copy of genomic DNA sequence, complementary to IL6 mRNA. The construct was copied with T7 polymerase following a protocol provided by the supplier (Stratagene). Two μg of plasmid DNA were suspended in a 50-μl reaction volume, containing 40 mM Tris-HCl, pH 8, 8 mM magnesium chloride, 2 mM spermidine, 50 mM sodium chloride, 0.4 mM each of ribo-ATP, ribo-CTP, ribo-GTP and 40 μM of UTP, 150 μCi of [^{32}P]UTP (400–800 Ci/mM, Amersham Corp.), 30 mM dithiothreitol, 50 units of RNasein (Promega) and 10 units of T7 RNA polymerase (Stratagene). The copying reaction was carried out for 45 min at 37 °C. The reaction mixture was then treated with 20 units of RNase-free DNase I (Stratagene) for 15 min at 37 °C. The reaction product was freed of unincorporated nucleotides by two cycles of addition of an equal volume of 4 M ammonium acetate and precipitation with 2 volumes of ethanol (27). The final T7 polymerase product was dried and redissolved in 50 μl of H_2O. For the annealing reaction, 12 μg of total RNA (extracted from the LPS-stimulated macrophage-like cell line RM-SV1, Results) were combined with 1 μl of the T7 polymerase product and the RNA was denatured in a 30-μl volume containing 80% formamide for 10 min at 75 °C. Then 3 μl of 10 × PIPES buffer (100 mM PIPES, pH 6.4, 4 M sodium chloride, 10 mM EDTA) were added and annealing was performed for 16 h at 45 °C. The reaction mixture was chilled on ice, and 300 μl of RNase buffer were added,

[2] M. Hattori and G. H. Fey, unpublished results.

[3] M. Hattori, unpublished results.

containing 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 200 mM sodium chloride, and 100 mM lithium chloride. RNase A (Boehringer, Pancreatic RNase) was added to a final concentration of 100 μg/ml, and 1000 units of RNase T1 (Bethesda Research Laboratories) were added. Incubation was allowed to proceed for 30 min at 37 °C. SDS was added to a final concentration of 0.3%, and 10 μl of a proteinase K (Boehringer Mannheim) solution containing 10 mg/ml were added. The reaction mixture was incubated for 25 min at 37 °C, and finally the RNase-resistant RNA products were extracted once with phenol, precipitated and washed with ethanol, and lyophilized to dryness. They were resuspended in 6 μl of formamide sample buffer (95% formamide, containing 20 mM EDTA, 0.05% bromphenol blue (Sigma), 0.05% Xylene Cyanole FF (Eastman Kodak)), and analyzed by electrophoresis in a 6% polyacrylamide urea sequencing gel (27, 34) and autoradiography. RNase H experiments were performed as described (29, 30, 39).

Southern Blot Analysis of Genomic DNA—The analysis was performed as described (39).

RESULTS

Rat Peritoneal Exudate Cells Produce Two Discrete Classes of IL6 mRNA—Northern blot analysis of RNA extracted from primary cultures of adherent rat peritoneal exudate cells (PECs) with a cloned murine IL6 cDNA probe (32) revealed the following two size classes of IL6 mRNA: a "major" class of 1.2 to 1.3 kb and a "minor" class of 2.4 kb (Fig. 1). Judged by quantitative densitometry of the autoradiographs, the minor class was approximately five times less abundant than the major class.

Isolation and Sequence Analysis of Rat IL6 cDNA Clones—Total RNA was extracted from primary cultures of adherent rat PECs, stimulated by treatment in culture with LPS (see "Experimental Procedures"). A cDNA library was constructed in the vector pGEXII (31) and screened with a cloned murine IL6 cDNA probe (32). Fourteen candidate cDNA clones were isolated from approximately 60,000 colonies plated and analyzed by restriction fragment length analysis (agarose gel electrophoresis). Further work was performed with clone pRIL6C.91, which carried the longest cDNA insert. The insert of this clone (1,055 bp, Fig. 2), covers most of the 5′-nontranslated region, the protein coding region, and all of the 3′-nontranslated region. The coding sequence specifies an open reading frame of 211 triplets (Fig. 2). The attachment site for the poly(A) tail was deduced by comparison of cDNA and genomic DNA sequences (see below). From the first cDNA library, no clones representing the 2.4-kb mRNA species were obtained, but subsequent screening of a second library led to the isolation and sequence analysis of such clones (see below).

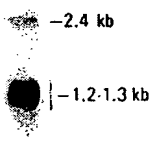

FIG. 1. **Primary cultures of adherent rat PECs produce two IL6 mRNA species of distinct size.** Primary cultures of adherent rat PECs were produced and RNA was extracted as described under "Experimental Procedures." Twenty μg of total RNA were electrophoretically separated in an agarose formaldehyde gel and transferred to nylon membranes. The blot was hybridized with a cloned murine IL6 cDNA probe (32). Size evaluation was made by comparison with the mobilities of RNA size markers (not shown).

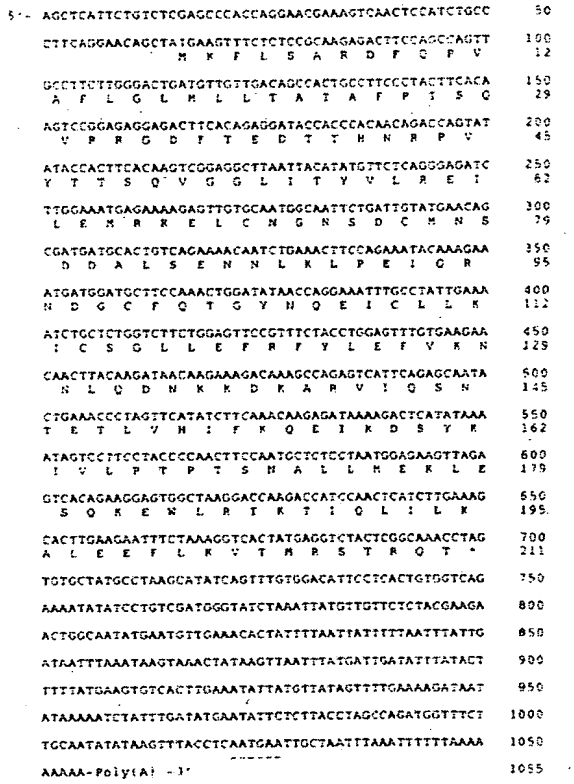

FIG. 2. **cDNA sequence and derived amino acid sequence of rat IL6.** The cDNA sequence and the derived amino acid sequence of the insert of clone pRIL6C.91 is shown. The position of the polyadenylation site was deduced by comparison with the genomic DNA sequence. The polyadenylation signal AATGAA (see "Discussion") is *underlined*, the *asterisk* locates the translational stop codon (*TAG*). The block structure shown below represents the IL6 cDNA. A single large open reading frame (ATG–TAG) encodes a precursor protein with 211 amino acids. The *black box* corresponds to the mature protein with 187 amino acids, the *dotted box* to the signal peptide with 24 amino acids, and the *white boxes* indicate the 5′- and 3′-*nontranslated regions, respectively. The numbers correspond to the* nucleotide sequence.

Properties of the Mature Rat IL6 Protein, Deduced from cDNA Sequence—The cDNA-derived amino acid sequence of mature rat IL6 is compared in Fig. 3 with the murine and human IL6 protein sequences. The rat and human cDNAs are 68% identical, and the corresponding rat and murine cDNA sequences are 92% identical. The rat and human protein sequences are 58% identical and the rat and murine protein sequences 93%. Murine, rat, and human IL6 contain 4 cysteinyl residues in conserved positions, and rat IL6 has an additional cysteine at position 88 (Fig. 3). The rat IL6 sequence carries no obvious *N*-glycosylation sites. The assignment of the NH_2 terminus of mature rat IL6 is based on a comparison with the known NH_2 termini of murine IL6.

Development of Stable Mammalian Cell Lines Expressing Rat IL6—To demonstrate that the cDNA clone pRIL6C.91

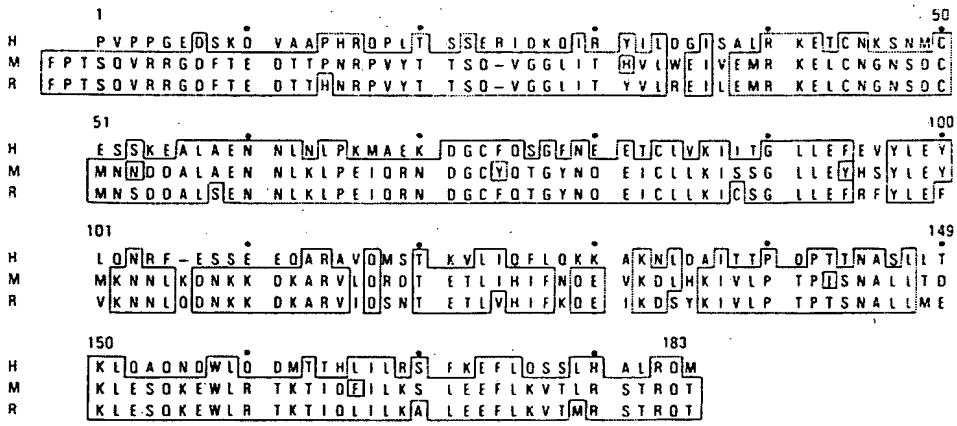

FIG. 3. **Comparison of human, rat, and murine mature IL6 amino acid sequences.** The human and murine sequences are as published (2, 32, 33). The NH_2 termini of murine and human IL6 have been determined by sequence analysis of the purified mature proteins (2, 43, 44). The NH_2 terminus of rat IL6 is deduced by sequence comparison with murine IL6. Identical residues are *boxed*. Gaps (*dashes*) were introduced as suggested by the computer program BESTFIT of the University of Wisconsin package (36) to produce optimal alignments.

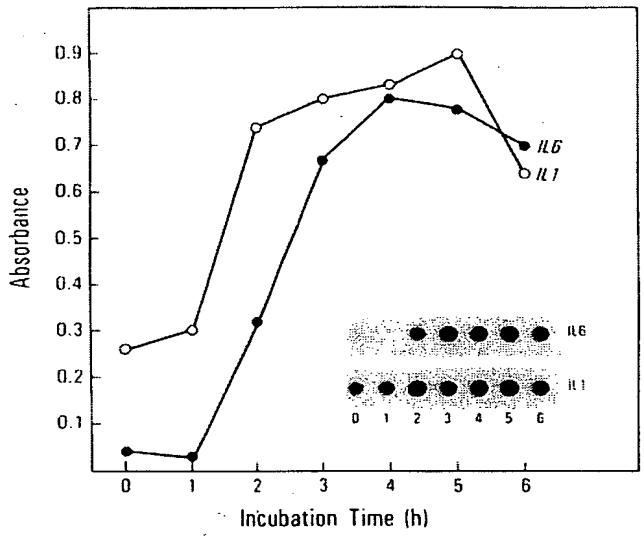

FIG. 4. **Induction of IL1 and IL6 mRNA levels in the cell line RM-SV1 by treatment with LPS.** RM-SV1 cells were grown in the presence of 0.5% fetal bovine serum. They were incubated for various time intervals with 1 μg/ml of LPS from *E. coli*. Total RNA was then extracted and aliquots of 10 μg of RNA were deposited on nylon membranes and covalently fixed by UV irradiation. Duplicate filter sets were separately hybridized with a nick-translated rat IL6 cDNA probe (pRIL6C.91) and a murine IL1 cDNA probe (51). Autoradiographs were evaluated by automated quantitative scanning densitometry. *Open circles*, IL1 mRNA; *closed circles*, IL6 mRNA.

codes for biologically active IL6, it was used for DNA transfection into mammalian cells. The transfected cells secreted significantly more biologically active IL6 than the untransfected parental cells. To this effect, the cDNA insert of clone pRIL6C.91 was subcloned into the expression vector pcDL-SR296 designed for high level expression in mammalian cells (45). DNA of this subclone, called pRIL6C.92, was transfected into murine L cells and human HeLa cells. Cotransfections were performed with a neomycin (G418) resistance marker, and G418-resistant colonies were selected. Stable cell lines were established by reiterated rounds of single cell subcloning, and the lines MLTK-RIL6.92 and HeLa-RIL6.92 contain stably associated rat IL6 cDNA sequences as judged by their ability to produce constant levels of IL6 activity (see below) for over 20 passages.

For comparison, an immortalized rat PEC-derived cell line, was also established. To this effect, primary cultures of adherent rat PECs were infected with the DNA tumor virus SV40 at a multiplicity of approximately 0.1 plaque forming units/cell. Foci of growing cells developed 2 weeks after infection and well-isolated foci were expanded into cell lines by multiple rounds of subcloning. The cell line RM-SV1, established in this manner, displays macrophage-like cellular morphology and other traits characteristic of highly differentiated macrophages. In particular, the mRNAs coding for IL1 and IL6 can be induced by stimulation of this line with LPS (Fig. 4). IL6 mRNA levels were increased 20-fold with a maximum at 4 h after stimulation, and IL1 mRNA levels were increased 3.5-fold with a maximum at 5 h.

TABLE I

Accumulation of biologically active IL6/HSF in various culture supernatants

IL6/HSF activity was assayed with the FaO/HB3 cell line using the luciferase assay (Experimental Procedures).

	Luciferase activity[a]	IL6 activity[b]
RM-SV1	2,882	110
RM-SV1 + 10 μg/ml LPS[c]	18,964	873
MLTK[d]	326	11
MLTK-RIL6.92	2,114	95
HeLa[d]	487	19
HeLa-RIL6.92	3,781	175
rhu IL6[e]	2,317	100
rhu IL6	7,000	300
rhu IL6	15,092	750

[a] Light counts integrated over a 30-s interval in a standard assay (see "Experimental Procedures") normalized for 200 μg of protein extract of FaO/HB3 cells. Basal level α_2-macroglobulin promoter activity obtained by culturing FaO/HB3 cells with media alone (696 counts) was subtracted from all activity values in this column.

[b] IL6/BSF-2 units/ml, as determined from calibration with recombinant human IL6.

[c] Treatment with LPS for 20 h. All values in this table are activities accumulated in a 20-h period.

[d] Control cell lines transfected with neomycin resistant vector only.

[e] Recombinant human IL6.

The cDNA Clone pRIL6C.92 Codes for Biologically Active IL6/HSF—Culture supernatants of the cell lines MLTK-RIL6.92 and HeLA-RIL6.92 as well as of LPS-stimulated RM-SV1 cells and corresponding controls were assayed for the presence of biologically active IL6 by using the indicator cell line FaO/HB3. This line is a sensitive tool to monitor the HSF, function of IL6. It converts an IL6/HSF input signal rapidly into luciferase activity, which can be measured with high precision (see "Experimental Procedures"). Using this

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   1  AATTCTTCAA ATGGGAAACC TGTCAAGATA CAGAGAAAAG AATGTATAAT GGTAGATAGA GTTTAGAGTT TAGAGTTTAG AGTTACTGTG GTAATATTTT
 101  GGTTTAAGAA GAGTGTTTTT CTCCCATAAA CAAATGCCAA TCAAAGGCAG TGGTGCTAAT AAAGATCCAT GCTGGTGACA TACATACAAA GGAACAAGAC
 201  AGAATCACCC TGCCTTCCAA ACCTTGAATA CTAGAAACTT AAGCTCTGGG GTTGTCAAGG CCATTTATGC TTGAGAATTA TGCTCAGAAA CTTGAAGGAT
 301  GATGATATGT ATAGACTGTT AATTCTCCTA TAAAAAAAAC TTAGCAGAAT ACTTTGGGAA AAGTCTAGGA GTAAAGCACA GCTGGCTAAT TAGTGAGTAA
 401  GGGACCTAGT TTGAGTATGG CTTTGTCTGC GTGTGTGAAT GTGCGTCACG TATATTCAGT GCCAACTGAG GCTATAAGAG GGCATCAGAT ACCCAGGAAC
 501  AAGAGTTACA GATGATTTAG AACCACGATG TAGAAACTGA ACCCACATCC TCTTGAAAGA GCAATAGATG ACCTTAACGT CCAAGTCATC TGTTCAGCTC
 601  CTCATAATGA ATTTTCAATA GCACCACCAA TCTCATTTCT TTAGGGATAA AATGGTTCAG AGACTAACTT CATCCTTGGT ACAGTAAGCT GGTTGTTCTC
 701  CATAGTAACT TCACAAGCAA GGTCCCAGGT GAACTGATAG TTATGCCAGA AAAGCTCCCT GAGGGAGAAG TTCCAGCTCT TAAAAATAGT AGAGTTGGTG
 801  CTTACTCTTT CTTAGTTATC TTTACGGCAG CTCTGTATGA TGACCTAGTA TTATTTTCCT GTTAAAGAGT AAATAAACAA GAACACAGAT CTTGGGCTAA
 901  TTTAGCCACT AATAAACAGC TAGCAAATGG TGGCATTGGA CTTCTAACCC AAATTATTAC TCAAAAGGCT ATCGTCTCCA CATGTACCCT GCCCCCACCC
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1201  TTACTTTCCT CTGTTGTCTT GTTTGAACAT TTTATCATGG ATGCATGCCT CCTACTTAAA GAGCATTTTT TTAAAAATTG AAAACAGAAA TTTGAATGTT
1301  GTAGTTAGTC TAACAGTCAA GCTTATTGAG TCATTTAAAA ATTGTTAAGT GGAGAAAAGA AACTCTATAC CTTAGAGACC TGAGAGAACA CATGGCAGGG
1401  AGTAGGGGAA GTAGAGACAT ACTGAGTTCC AGGCTATTTC AGCTGCCATT TTAAGGTCCA AGGGCACTAA AAGTAAAACC ATCCTGCTTT CTCTCCCCCA
1501  TTTCCATTTT CAACTACAAT CCCCTAGAAC CTATGTGAGG ACTAGTGCCT CACACAGTGA AAGAATGGGA AACCTCACTT CTTCAATAGG CAGTTTCTCG
1601  TTTATGTACA CAAACAAGAG TCCACCCATA TTTTAACTGT CCCAGCAACA GTCATTGCTT TCAGAGTCTT AAGGCAATCT GCAAAGTGAA GAAACTGATT
1701  TCGATGCTAA GAGTCTGATT TGTAAATAGT TTTGGGTTTA TATACAGAGC CTACTTTCAG CCTGTAATCA TTTTGAGTGC TATCTAGGTA TCTGGAGACA
1801  GGTGGACAGA AAACCAGGGA CTAATCTGTA AAATAAATTG AACAAAGGGA AGAAGTCTGT TTAAGCTTGA CCCAGCCTAG AAGTCTCGGA CATTAGAAGG
1901  GTCATTCTGA GTGAGACATG CCACCTTTAG ATTCAAATCC TGTCACCCAG TTAAAGGGAG CTTCAAACAT CTAGCTACGA TACAACGAGG TCCTTCTTTG
2001  CTGTAAACCC TGAATAAACG TAAGGACTGC AACAAACCTT CAAGTCTCCT TGCATGACCT GGAAATGTTT TGGGGATGTT CTGGCAGCAG TGGGATCAGC
2101  ACTAACAAAT AAGGCAACTC TATTACAGAA ACTAAAAGTC TTACTAGTAA GATGGCCAAG AGACCACTAG GGAGAAATCA GAAAGTGGAC TTGGACTTGA
2201  GAGGAAACCA GGAGTGCTTG ATGACAGAAG ATATTTCTGT TCTTCACTCC ATAGTAGCCC ACCTGGCAAC TCCTGGAAAC AACTGCACAA AATTTGGAGG
2301  TGAACAAACC ATTAGAAGCA GCTGGTCCTG ATAACATGCA GGAAGAACAA GCAATATGCA GCAGGCATCA CTACTGTCTG TGTGACTCCA GGTCAGGTGC
2401  TGGGGGTGGA GTGGGAGTGG GAGTGTGTGT CTTTGTATGA TCTGAAGAAA CTTAGGTCAG AACATCTTTA GATTCTCACA GACATGAAAA AATTAGAAGA
2501  GTAAATCCTG GCCTCTTATT AATTCATGAG TGTTTGTGTG TGCGCACATG TGTTTAAATA ACATCAGCTT TAGCTTCACT TTCTCCTTAT AAAACATTGT
2601  GCATTTCAGT TTTTCCCCCT ATCAAGTGCT CAAGTGCTGA GTCACTTTTA AAGAAAGAAA AAGAGTGATC AGGCTTCTTA AGGATAGCCT CAAGGATGAC
2701  TTAAACACAC TTTCCCCCTC CTAGCTGTGA TTCTTTGGAT GCTAAATGAC GTCACATTGT GCAATCTTAA TAAGGTTTCC AATCAGCCCC ACCCACTCTG
                                                                                                    TATA
2801  GCCCCACCCC CACCCTCCAA CAAAGATTTT TATCAAATGT GGGATTTTCC CATGAGTCTC AAAAGTAGAG AGTCGACTCC CAATAAATAT GAGACTGGGG
          m   M
          ↓   ↓
2901  ATGTCTGTAG CTCATTCTGT CTCGAGCCCA CCAGGAACGA AAGTCAACTC CATCTGCCCT TCAGGAACAG CTATGAAGTT TCTCTCCGCA AGTAAGTGAA  Ex1
3001  GGCAGTTTCT CGCCCTCTGG CGGAGCTATT GAGACTGTGA GAGGAGTGTG AGGCAGGGAG CCAGCCAAGT GGGTTGGCTA GCAGCCAGCA GCCAGCATCA
3101  GGCGCCCAGC TGGGCTATTT TCTCATTTGC TTCTTTCCCT TTCCTCCACG CAGGAGACTT CCAGCCAGTT GCCTTCTTGG GACTGATGTT GTTGACAGCC  Ex2
3201  ACTGCCTTCC CTACTTCACA AGTCCGGAGA GGAGACTTCA CAGAGGATAC CACCCACAAC AGACCAGTAT ATACCACTTC ACAAGTCGGA GGCTTAATTA
3301  CATATGTTCT CAGGGAGATC TTGGAAATGA GAAAAGAGGT GGGTAGGCTG TGAAAGTGAT GACGGCCCAA TGTGGGCATC CATTCATTCT CTTTGCTCCT
3401  GAATTGGGAA TTCTCTGCTG GGTTCTAGAG CCCTTTGGAT TTGAAGCTAA AGATCAGACT AGACTTGTAT TCTCTCTCTC TCTTCTCTTT GCTGGTTTTG
3501  GGTGGGAGTT GTGTGTGTGT GGTTCCTTCC CTGTCTGGAA GATACAGAAT GTGAACTGCA TTTCTAGAAA ATTCACAGTG GACCATTCTC AGTTCCAATT
3601  TAGTTGTTGT TTCTTGGGTA GGGATTCCTT TTTCTTTATC AAAGAGTAAG ATCTCTTGGC CTTCATTTGT CCTTTGAACT CTACAAGGAC CAGAAAGTGC
3701  TTTTTGGCTG AATGTAGTAA GCTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTAATCA GGATGCTCCA GGGTCAGCCC
3801  AGCTAAGATG CATATTGTAT AGAGCCTAAT AAAGTACTAA CATAAAAAAA AATAGATAAG GCTGGGCATA ATGGTGGATA CCTCCCAGCA CTTGGGAGGC
3901  ACAAGCAGGA AGATTTTTGT GAGTTAGAGG AAAGCTCAGT CTACATATTG AGTTCCAGGA TACCCAGGAC TACATTGGGA GATCCTTTCT CAAAAACAAA
4001  ACAAAATAAG AAAACAAAAA ACCCAAAACC AACCAACCAA CCAACCAACC AACCAACCCT ACCTAGATTA AAATATCTCA CATCTTTAAA TGCAAAGTAT
4101  TTTGACTCCG CTTGACAGAA AGAAAAAAAA AACACAGTCA AGATTAATAG CCTATTCTCT AGGTCTGGGT GTGTAGAATC AACTTTTTAA TCTTGGGAAG
4201  ATTCCCAATT TATTTAGTAC TCTAATTATG GAGTTAGAGA TGATGGCACA GACCCTTCCA GATGGAATAT TCTCTGACTT AAATTTATTT GCCCCAAAAT
4301  ATCATCACAA TTTCTGTCCT TTACTAAGCT CCTACTCTAT GCTGGGCAGT TTTATCATCG AATCGTTGAA ATAAATGTAT AAGAGGGACT TCAATAAGTC
4401  TTTATTTAAA CATAGGCAAT ACCTAAGTAA GAGTACAAAG CTTGTAGTCA AAAGAGAGCC TGGGACTGAG CCTAAGGGAC CCTTTTTATT TATTGGGCTG
4501  CTTAGTAAGG TTCTAAGAAG GTGTAGCTTC CCTTTTCTTA ATGTGTCCTG GCTATGGAAG AGCATGACTT GGCCCTCTAG TGGTGTTTGT TGTAGGAGTA
4601  CTTAAGTGCT CATTCTGGTA ATCTTTTGCA GTTGTGCAAT GGCAATTCTG ATTGTATGAA CAGCGATGAT GCACTGTCAG AAAACAATCT GAAACTTCCA  Ex3
4701  GAAATACAAA GAAATGATGG ATGCTTCCAA ACTGGATATA ACCAGGTAGG AACTTGTCAC ATTTACTTCC AGCAATTCTT CAGCCAATGT TCTCTCTTGC
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FIG. 5. **Sequence of the rat IL6 gene.** The sequence was determined as described under "Experimental Procedures." Exons are *underlined* and labeled in the right margin. The TATA box is *underscored* and the positions of the minor and major mRNA cap sites are indicated by *arrows*, labeled *m* and *M*. The extended version of exon 5 is shown. The polyadenylation signals used for the major and minor mRNA species are indicated by *dashed lines* above the sequence at nucleotides 7479 and 8691.

```
4801  ATTGATACCT GCATATATAC AGTCTCAGTC AGGTAACAAA GATGGGGAAG TACATCCAAC ACCACTTGGA TGTAACTGGC CTACAGGAGC TTGCATACAA
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5001  CCTCTTGGCT TTGAGGTGTT AGATGATTAA AAAAGATTTT TTTTCTCTAA AAAAATGAAA GAGTAGTTAT AAAAAATGTT ATGTAAATGT CTTGAGGAAA
5101  GTAACTTACA GCTTTAATTA AAAAGCAATT TCTTTGAACA GGTAAAGTCC CACTACACTG TTAAATGTTT AGCAAGTTTA GCTAGTTCAT GTCTAGTACA
5201  GTAGTTTATG AGCCATTCTC TTCTCTGGTT GCCCCTGGAA GGATTCAGGC CTACTCTCCC TACTGAGGAC ACTCTTCATA CCCCTCTCCT TCCAGCCAGT
5301  GATATAATCT GCATGGGTTT CTTATTTCTT AAAAGCAGGT CTCAGGTCAG CCCAGATAAG AACAAGAGTA CAGGAAGTGC ACTGGTGGGT AGAAGGCAAG
5401  GAGTCGGGTC AGAAGTAGAT TCTCACTATC ATACTCTGGG ACCCAGGGGG AAATATTAAG TGGTGCCTTT CTCTACTTGT CCAGATAATT AAAGGAGGTC
5501  AGCTGATGCT GCCTATTGCC CAATTCTGTA GAAAATGCTG AACAGCAGGA ATCTTATTCT CAAATTGAGT CTATCTCCTA GAAAGAACTG ATATCTCCTT
5601  CCATTTTACT TATAGGAAAT TTGCCTATTG AAAATCTGCT CTGGTCTTCT GGAGTTCCGT TTCTACCTGG AGTTTGTGAA GAACAACTTA CAAGATAACA  Ex4
5701  AGAAAGACAA AGCCAGAGTC ATTCAGAGCA ATACTGAAAC CCTAGTTCAT ATCTTCAAAC AAGAGGTGAG TGCGTCCCCA TCTCTCATGC AGTGTAGGGA
5801  AGAGGTGCTA GGAGAAAACA CCCAGCATCT CAGGGTAGCA GCACTTTTTC CAGATAGCTG CTCAAAAGGG AGAGTCTGAG CAACAGTTCT TTGACTGTAA
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6001  CTTAAGAGAT AAGAAAGCCT TAACAGACAA AAGGAAGCAT ATCCAACCTT ACACAGTCAT CCATTATGAG AACATAGAGC ACAAAAGTGA CTTTAATATT
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7001  CATATAAAAT AGTCCTTCCT ACCCCAACTT CCAATGCTCT CCTAATGGAG AAGTTAGAGT CACAGAAGGA GTGGCTAAGG ACCAAGACCA TCCAACTCAT
7101  CTTGAAAGCA CTTGAAGAAT TTCTAAAGGT CACTATGAGG TCTACTCGGC AAACCTAGTG TGCTATGCCT AAGCATATCA GTTTGTGGAC ATTCCTCACT
7201  GTGGTCAGAA AATATATCCT GTCGATGGGT ATCTAAATTA TGTTGTTCTC TACGAAGAAC TGGCAATATG AATGTTGAAA CACTATTTTA ATTATTTTTA
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7501  TTTTAAAAAA AAATCTTTGT GATGTATTTT TTAAATTCTG TTTTATTTAC ATTTTAAATG TTATCCCATT TCTCGATTTC CCATCCAGAA ACCTGATATC
7601  CCAACCTCAT CCCCTAGTCC CCCACCCTCT CACTCCCCCC TAACCCCCCC TCCGCTTCTA CCCACCCACT CCCTTCCACC TCTCCACCCT GACATTTCCC
7701  TGTACTGGGG CATCGAGCCT TGACAGGACC AAAGGCCTCT CCTCCCATTG ATGTGGACAA GGCCATCCTC TGCTACATAT GCAGATGAAG CCATAGGTCC
7801  ATCCCTGTGT TCTCTTGGGA TAGTGGTTTA GCTCTGGGAG CTCTGGGCTG TCTAGTTGGT TGATATTGTG TTCTTCTTAT GGGGTTGCAA ATCCTTTCAG
7901  CTACTTCAGT CGTTTCTCTA ACTCCTCCAT TGGAGACCCT GCTCTCAGTT CAATGGTTGG CTACGAGCAT CTGCCTTTGT ATTTGTAAAG CTTTGCAGAG
8001  CCTCTCAGGA GACAGCTATA TCAGGCTCCT GTCAGCATGC ACTTCTTTCA TCTGCGATAT TGTTTGGGTT TGGTGGCTGT ATATGGGATG AATCCCATGT
8101  GGGGCAGTCT CTGGATGGCC TTTCAGTTTT TCAGTCTCTG TTCCACACTT TGTCTCTATA TTTCCTCCTG TGAGTATTTT TTTCCCCCTT CTAAGGTTTG
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8401  AGTTTCACCT GACTCCCAGG AAACCAGGGT GGTCACTAAC CCATAGCCCT CCATAGATTT GTGACTATCA GTCACATAAG GGCAACACCC CAAGCCCCTT
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8601  TCCCAGACAC TCCTCCCTGC AAAAGGTATT TAATCTCAGG CCCACTCTGA GAAGTGGGAT ATGGTTTTAT TCATCCACTT TCTGCCATGA CAATAAACAC
8701  CTTGAAACCA TGGACTGTCT CTTTTCATCG GGATCTGACA TGGGGAGTTA CAAAGAAGGC CTTTGCCTAC AGTGCCACCA TCTAATCTCC CAAAGAAAGC
8801  CTCCTGCGCT CTCAGCAGCA GCAACTGCCA AGCTCAAGCA TACTGCTGAA CTGCTGACCA GCCAAGGCTA CCCCAGTGGG ACCAGCTGCA GTGTCCCCTC
8901  TTTCTCCCTC AGCACTGGGC TAGATCCACT TCCACAGCCC CCCACTCCAT TCTCAGCTCT TCCTCTGCAT CCTAGTGGTT CCCAGATGCC CAAGAGCCTG
9001  AGAACTTGAT GACCCTGGCC TCCTTGCAGG CCCAGGGATG GTGAGTCAGC TCTGGCTATG TCTGGATCCC AGACTGTGCT TCCCCACCTC CTGGAACATT
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9201  AGGGAGCCCC ACAATTAATT CTACAGGTTA TCTCACTGAG AATGATATCT TCCATCTATT TGCCTAAGAA TTTCATGAAG TCATTGTTTT TTTATTAGAT
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9901  TCTTGAACTT CATGTGGTCT GTGGATTGTA TCTTGGGTAA TCTGAGCTTT TGGGCTATTT TCCACTTATC AGTGAGTTCA TACCATGTGT GCTTTTTTGT
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```

FIG. 5—*continued*.

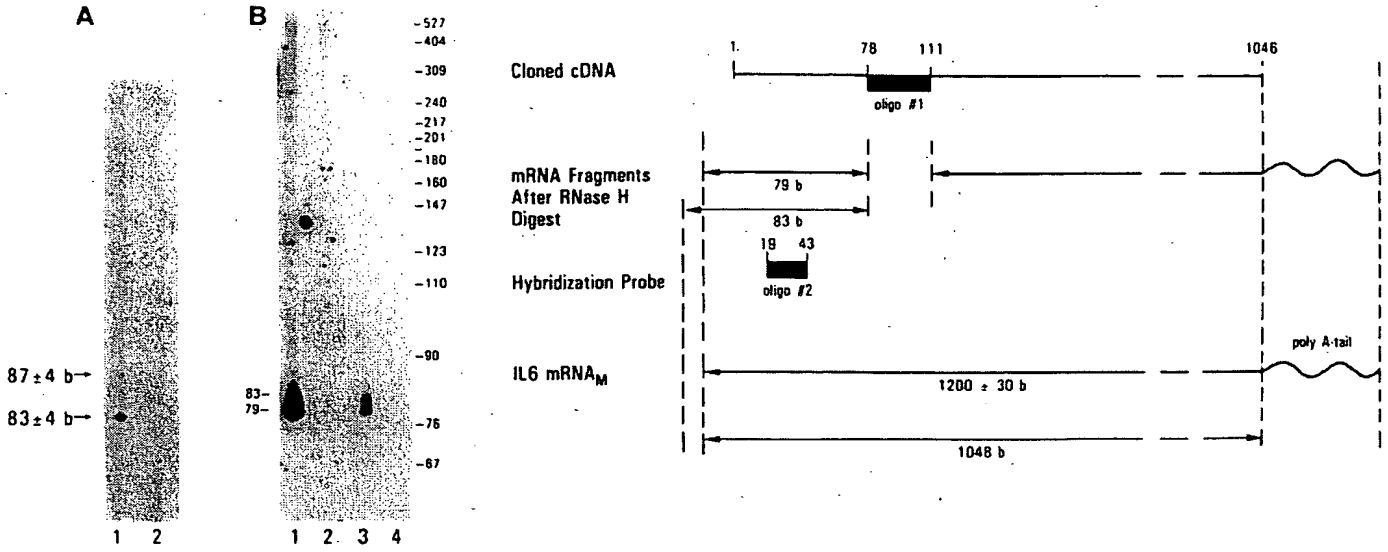

FIG. 6. **Mapping the 5′ ends of IL6 mRNA.** *A*, mapping by RNase protection. Internally labeled RNA copies of a genomic DNA fragment containing exon 1 were produced by subcloning the fragment into a bluescript vector and copying each strand separately into a single-stranded RNA (see "Experimental Procedures"). *Track 1*, the probe complementary to mRNA, and *track 2*, the probe of the same strandedness as mRNA were annealed with non-radioactive mRNA from LPS-stimulated RM-SV1 cells. The reaction products were treated with a mixture of pancreatic RNase and RNase T1, and RNase-resistant fragments were analyzed by electrophoresis on a sequencing gel. The two resistant bands labeled by *arrows* define the map positions of the minor and major start sites (*m* and *M* in Fig. 5). The size marker used was radioactively end-labeled *Msp*I-digested pBR322 DNA fragments, previously calibrated against RNA markers. *B*, mapping by RNase H experiment. Oligonucleotides 1 and 2, both complementary to mRNA, were synthesized based on the cDNA sequence. Oligonucleotide 1 was annealed with RNA extracted from LPS-stimulated RM-SV1 cells and from inflamed rat livers, and the reaction product was treated with RNase H, generating RNase H-resistant fragments as represented schematically. The resistant fragments were electrophoresed in a polyacrylamide sequencing gel and transferred to a nylon membrane. The blot was hybridized with radiolabeled oligonucleotide 2 to reveal the size of the resistant fragments. *Tracts 1* and *2*, 4-day exposure of the autoradiograph; *tracts 3* and *4*, 1-day exposure. Reactions analyzed in *tracks 1* and *3* were performed with 5 μg of poly(A)$^+$ mRNA from LPS-stimulated RM-SV1 cells, reactions in *tracks 2* and *4* with 10 μg of poly(A)$^+$ mRNA from rat liver, 18 h after induction of an experimental acute-phase reaction by intraperitoneal injection of complete Freund's adjuvant, as described (29). The sizes of the hybridizing fragments at 79 and 83 bp define the positions of the minor and major cap sites (*m* and *M* in Fig. 5). The length of the major mRNA species is calculated to be 1048 bp, without the poly(A) tail, as shown in the bottom line (mRNA$_M$). The minor mRNA species is four nucleotides longer and not shown for simplicity.

indicator cell line, calibrated with known standards of recombinant human IL6, we determined that RM-SV1 cells accumulate 110 IL6/BSF-2 units/ml of culture medium in 20 h, and that treatment with 10 μg/ml of LPS leads to an 8-fold stimulation in 20 h (Table I). Mouse LTK and HeLa cells transfected with neomycin-resistant vector only produce low background levels of an endogenous IL6-like activity that score in this assay with 10 IL6/BSF-2 units/ml and 18.5 units/ml, respectively, accumulated in 20 h. The line MLTK-RIL6.92 accumulates 9-fold increased IL6/HSF amounts in the culture medium, and HeLa-RIL6.92 cells accumulate 9.4-fold increased levels in the same time period. Thus, the transfected cell lines accumulate significantly increased levels of IL6 in their culture medium, and we conclude that the cDNA clone pRIL6C.92 codes for biologically active rat IL6, at least for the HSF activity of IL6, designated IL6/HSF.

Isolation and Sequence Analysis of the Rat IL6 Gene—A set of three genomic DNA clones contained in phage λ vectors were isolated from a rat genomic library by screening with the cDNA clone pRIL6C.91 (see "Experimental Procedures"). A restriction enzyme cleavage map for the enzyme *Eco*RI was produced, and two contiguous *Eco*RI restriction fragments of clone λRIL5G-9 (a 3.4-kb fragment and a 6.66-kb fragment) were randomly subcloned into M13 phage vectors and sequenced. The resulting 10.067-kb sequence (Fig. 5) contains the entire 5.8-kb rat IL6 gene as well as approximately 2.9 kb of 5′- and 1.2 kb of 3′-flanking sequence. The exon/intron boundaries were located by comparison of the cDNA and gene sequences. The 5′ end of the mRNA was mapped both by RNase protection and by RNase H experiments (Fig. 6). The result of both experiments demonstrated the existence of two mRNA size classes, a minor abundance class starting at nucleotide 2903 in the genomic sequence (*labeled m* in Fig. 5) and a major abundance class starting at nucleotide 2907 (*labeled M* in Fig. 5).

The 2.4-kb mRNA Species Contains Additional Sequences in the 3′-Nontranslated Region—The experiments performed to map the 5′ ends of the RM-SV1 cell-derived IL6 mRNA did not provide evidence for additional transcription start sites located further upstream in the gene. Therefore, the 2.4-kb mRNA species could either be derived from a second gene, or contain additional intron sequences or additional sequences from the region located to the 3′ side of the polyadenylation site used for the 1.2-kb mRNA species (nucleotide 7505 in Fig. 5). To test for additional intron sequences, intron-derived oligonucleotide probes were synthesized. Northern blot hybridization with intron probes was performed, but the 2.4-kb mRNA species failed to react.[4] However, the 2.4-kb mRNA species selectively hybridized with a 1.22-kb *Sac*I/*Bam*HI-

[4] W. Northemann, unpublished results.

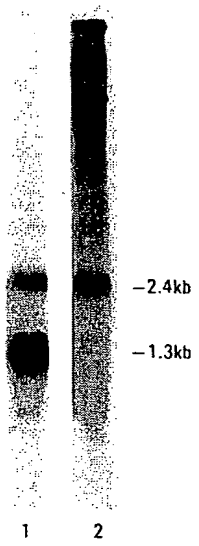

FIG. 7. **The 2.4-kb minor mRNA species is extended at the 3′ end.** RM-SV1 cells were stimulated for 18 h with 10 μg/ml of bacterial LPS, and 10-μg aliquots of total RNA were separated by electrophoresis. After transfer to nylon membrane (GeneScreen) the filter was hybridized either to radioactively labeled rat IL6 cDNA (*track 1*) or to a 1.22-kb *Sac*I/*Bam*HI-genomic DNA fragment (*track 2*) located 3′ of the polyadenylation site of the major mRNA species (at 7505 in Fig. 5) in the gene. Hybridization in the high molecular weight range in *track 2* is probably due to L1 repetitive sequence elements contained in the radiolabeled genomic DNA probe fragment.

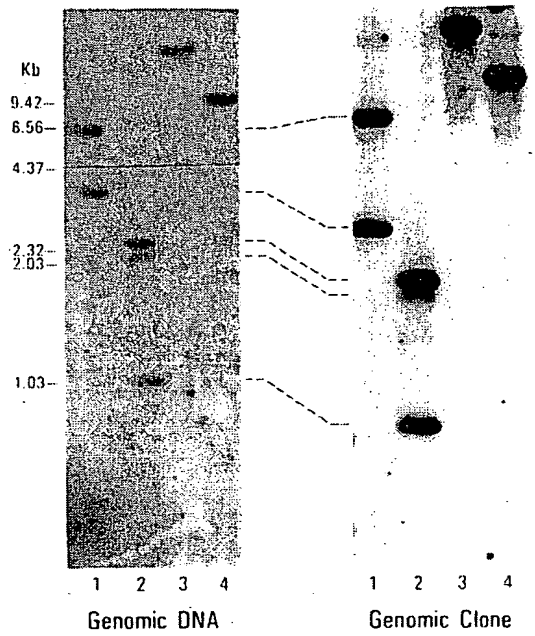

FIG. 8. **The rat IL6 gene is a single copy gene.** Rat genomic DNA (*left*) and DNA of the genomic clone λRIL6G.5 (see Fig. 9, *right*) were digested with *Eco*RI (*lane 1*), *Hin*dIII (*lane 2*), *Sac*I (*lane 3*), and *Bam*HI (*lane 4*). Twenty μg of the genomic DNA digest and 1 μg of the phage DNA digest were loaded in each lane and separated by electrophoresis in a 0.9% agarose gel. The DNA was transferred to a nylon filter (GeneScreen) and hybridized with the IL6 cDNA clone pRIL6C.91 in 6 × SSC at 63 °C for 36 h, as described (39). The cDNA probe was radioactively labeled by nick translation using the random primer method (Random Priming Kit, Amersham Corp.). DNA sizes were evaluated with reference to size markers that were carried in the same gels (not shown). Fragment sizes in kilobases are indicated in the *left margin*. Equivalent fragments in both gels are connected by *broken lines*.

genomic DNA fragment derived from the gene region downstream of position 7505 (Fig. 7). Therefore, the 2.4-kb mRNA species must contain additional sequences that are not present in the 1.2–1.3 kb species and which are derived from the gene region downstream of nucleotide 7505 in Fig. 5. To establish this fact, a second cDNA library of greater complexity than the first was produced from poly(A)$^+$ mRNA isolated from LPS-stimulated RM-SV1 cells. This library was screened with the cDNA clone pRIL6C.91, and positive isolates were counterscreened with the 1.22-kb *Sac*I/*Bam*HI-genomic DNA fragment, representing genomic sequences located to the 3′ side of nucleotide 7505. Several isolates hybridizing with both probes were obtained. The isolate with the longest insert was sequenced (data not shown). Its sequence was identical with the genomic sequence, and the polyadenylation site for this mRNA species is located at nucleotide 8708 in Fig. 5. Thus, the 2.4-kb mRNA species contains in its 3′-nontranslated region an additional segment of 1203 contiguous nucleotides beyond those present in the 1.2–1.3-kb mRNA species. The additional sequences are derived from the 3′-terminal region of the gene immediately downstream of nucleotide 7505 in Fig. 5.

The Rat IL6 Gene Is a Single Copy Gene—To determine whether both mRNA species are derived from a single gene or from two different IL6 genes, we counted the number of rat IL6 genes. Southern blot analysis (Fig. 8) revealed only one IL6 gene in the rat genome. Thus, the rat IL6 gene is a single copy gene, and the 2.4-kb mRNA must be derived from the same gene locus as the 1.2-kb species by use of alternative polyadenylation. Therefore, exon 5 of the gene exists in two sizes, corresponding to the 3′-nontranslated regions of both mRNA species (Fig. 9). Sequence analysis of the genomic region selectively present in the long version of exon 5 revealed the presence of a repetitive genomic sequence element of the L1 class, that is approximately 800 bp long and 72–80% identical with the reported rat and murine L1 consensus sequences, respectively (46–48).

DISCUSSION

Microheterogeneity in mRNA length for human IL6 was discovered by sequence analysis of variant cDNA clones differing at their 3′ ends.[5] The variant species do not differ sufficiently in size and/or are not of sufficient abundance to be detectable as separate bands by Northern blot analysis. Human and murine IL6 mRNA appears, as far as previously published, as a single band in the 1.2–1.3 kb size range on Northern blots. The presence of a separate 2.4-kb mRNA species 20% as abundant as the major species is a unique property of rat macrophages (Fig. 1) and of other rat cell types producing IL6.[4]

The cDNA sequence coding for the mature portion of rat IL6 is 92% identical with the corresponding murine sequence and 68% identical with the corresponding human sequence, while the degrees of amino acid sequence identity are 93 and 58%, respectively. Thus, the two rodent IL6 species are highly conserved, but the degree of relatedness between the human and rodent IL6 sequences is surprisingly low given the fact that recombinant human IL6 acts on rat liver cells and must therefore be recognized by the rat hepatocytic IL6 receptor. This is surprising because other cytokines such as G-CSF (9) do not act across species boundaries, although their degree of inter-species sequence conservation is higher. One might ex-

[5] W. Northemann, J. Gauldie, and G. H. Fey, unpublished results.

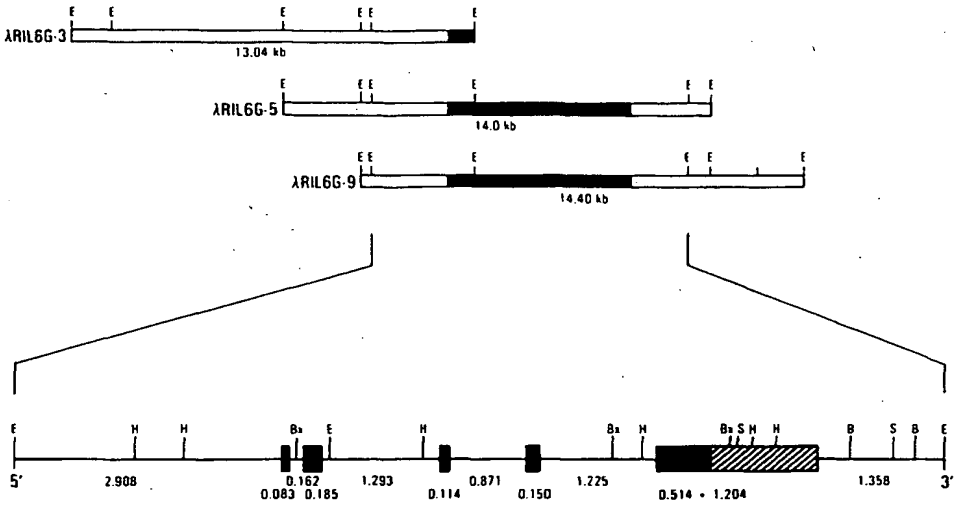

FIG. 9. **Exon/intron block structure of the rat IL6 gene.** The inserts of the three overlapping genomic phages λRIL6G.3, λRIL6G.5, and λRIL6G.9 are shown as *open boxes*. The insert lengths in kilobases are given underneath. *Eco*RI restriction sites are labeled (*E*). The transcribed portion of the gene is shown in *black*. *Bottom line*, expanded restriction map of the 10.06-kb sequenced portion of the gene (two contiguous *Eco*RI fragments), showing additional sites for the enzymes *Hin*dIII (*H*), *Bam*HI (*B*), *Sac*I (*S*), and *Bst*xI (*Bx*). Exons are given as *black boxes*; the extended form of exon 5, created by alternative polyadenylation, is shown as a *shaded box*. Exon and intron lengths in kilobases are given underneath.

pect to find the receptor-binding domain of IL6 contained in a part of the molecule that is conserved among the three species.

The assignment of the NH_2-terminal amino acid of rat IL6 (Fig. 3) is not based on analysis of purified mature rat IL6 but on comparison of the cDNA derived sequence with murine IL6. Mature murine IL6 occurs with two different NH_2-terminal residues, resulting from cleavage of the precursor polypeptide by signal-peptidases at two adjacent sites (43, 44). Since the rat IL6 protein sequence is identical in this region with murine IL6, we predict mature rat IL6 may similarly occur with two possible NH_2-terminal residues, provided the rat signal peptidases have similar sequence specificities as their murine counterparts.

Human IL6 has been reported to be both *N*-glycosylated and *O*-glycosylated, while the murine IL6 sequence lacks potential *N*-glycosylation sites, and *N*-glycosylation has not been detected for murine IL6 (32, 33). Rat IL6 is similar to murine IL6: the cDNA sequence predicts no *N*-glycosylation sites. Direct studies of glycosylation of the rat protein have not yet been reported. It is possible that rat IL6 carries *O*-linked carbohydrates. It will be interesting to investigate whether any one of the multiple functional properties of human IL6 are specifically dependent on *N*-glycosylation and whether rat and murine IL6 lack those properties.

The number of cysteinyl residues is conserved between murine and human IL6, and therefore, presumably both molecules have the same disulfide bond connectivity. The same 4 cysteinyl residues are also found in rat IL6, plus an additional fifth cysteinyl residue. It is to be expected that the 4 conserved rat cysteines will be engaged in the same disulfide-bond pattern as in murine and human IL6, and therefore the fifth rat cystein is expected to be unpaired. This would endow rat IL6 with the possibility to engage into an intermolecular disulfide bond or to block this reactive group by other secondary modifications. In the future it will be interesting to investigate whether rat IL6 has an increased propensity over human and murine IL6 to form dimers or aggregates with other molecules by virtue of this additional cysteinyl residue or whether it displays other functional particularities due to secondary modifications of this residue.

The cell line RM-SV1 is a convenient source of rat IL6 mRNA of constant quality. The line is believed to have originated by transformation of a macrophage with SV40, although we cannot formally exclude the possibility that it was generated by transformation of a monocyte or another cell type present in adherent primary PEC cultures. However, since macrophages and monocytes are the most abundant cell types among adherent PECs, and since the line has a macrophage-like morphology with ruffled edges and presents other traits characteristic of macrophages,[4] we refer to it as a macrophage-derived cell line. A characteristic property of primary peritoneal macrophages is their failure to divide in culture. The line RM-SV1 grows indefinitely, and therefore clearly differs from primary macrophages. It shares with primary macrophages the important property of being inducible by LPS. The line produces residual levels of IL1 and IL6 mRNAs and of IL6/HSF activity, and both mRNAs and the IL6/HSF activity are clearly inducible by treatment with LPS (Fig. 4 and Table I).

The cDNA clone pRIL6C.92 clearly codes for biologically active rat IL6 because both human and murine cell lines transfected with these rat cDNA sequences show an approximately 9-fold increase in secreted IL6/HSF activity over background. The levels of IL6 activity secreted by the transfected lines are in the range of those produced by nonstimulated macrophages (100–200 IL6/BSF-2 units/ml accumulated/20 h) and about 2-fold less than the levels found in a strain of primary human lung fibroblasts (Ven 8 strain), which accumulate 200–300 IL6/BSF-2 units/ml in 20 h.[6]

The exon/intron structure of the rat IL6 gene (Fig. 9) closely resembles those of the murine and human IL6 genes, as well as that of the human G-CSF gene (49, 50). The positions of exon/intron boundaries and the exon lengths are conserved in the protein coding portion of the gene, but the genes differ at the 5′ boundary of exon 1 and the 3′ boundary of exon 5, which are located outside the coding part. For the human IL6 gene, three cap sites have been reported (C1 to C3), with the closest site (C1) located 63 to 64 nucleotides 5′ of the translation start triplet, the second site (C2) at −86 to −88 and the third site (C3) at −176, and all preceded by conventional TATA-box sequences (49). In the rat gene only two cap sites were detected (*m* and *M* in Fig. 5), separated by only four nucleotides, located 67–71 bp upstream of the translation-initiation triplet, and preceded by a conventional TATA-box sequence. No sites located further upstream were detected by using mRNA prepared from the rat macrophage-derived cell line RM-SV1. We cannot exclude the existence of additional potential sites of transcription initiation for the

[6] M. Hattori and J. Gauldie, unpublished results.

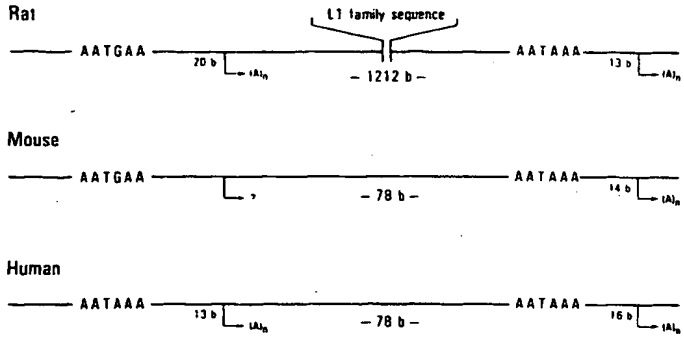

FIG. 10. **Comparison of polyadenylation sites used in the rat, murine, and human IL6 genes.** The AATGAA polyadenylation signal used for the major 1.2–1.3-kb rat IL6 mRNA species and the corresponding polyadenylation site $(A)_n$ with the distance between both in base pairs (*b*) are given in the top row at the left. The position of the inserted L1 repetitive sequence element (730 bp) is indicated. The canonical AATAAA polyadenylation signal used for the minor 2.4-kb mRNA species, the distance in base pairs (*b*), and the location of the polyadenylation site $(A)_n$ are shown. The distance between the two polyadenylation sites (1212 bp) is indicated below. The mouse genome contains two potential polyadenylation signals, but as far as it is known only the canonical signal AATAAA is used. The human genome contains a very similar arrangement as the mouse genome with two sites at a distance of 78 bp, and both are used.

rat IL6 gene that may only be used in cell types other than macrophages. Differential utilization of the three start sites of the human gene was reported in different cell types expressing human IL6 (49).

The presence of two different mRNA species differing by 1.2 kb in their 3′-nontranslated regions and generated by alternative polyadenylation is a unique property of the rat IL6 gene. The longer version of exon 5 of the rat IL6 gene contains a sequence of the L1 repetitive sequence family, located between nucleotides 7537 and 8337 in Fig. 5. This sequence is approximately 72 and 80% identical with the reported consensus rat and murine L1 sequence elements, respectively (46–48). Thus, the rat gene differs from its human and murine counterparts either by the insertion of this sequence element into the rat gene after speciation, or by the loss of this sequence from both the human and murine genes after speciation. The murine and human genes have highly conserved sequences in this area extending until a position equivalent to nucleotide 7536 in the rat gene (Figs. 5 and 10), and an independently occurring loss at the same position in both species is highly improbable. Therefore, it is almost certain that these sequences were inserted into the rat gene after speciation. While L1 sequence elements are not uncommon and frequently recombine, there are few instances, in which they have been reported to become part of a transcription unit and to remain present in a stable mRNA species (48). In this respect the rat IL6 gene represents a so far unique case.

We refer to the polyadenylation signal as the hexanucleotide sequence that is a variant of the canonical sequence AATAAA and that precedes the site of attachment of the poly(A) tail (the polyadenylation site) usually by approximately 13–20 nucleotides. The major 1.2–1.3 kb rat IL6 mRNA species is produced by using the polyadenylation signal AATGAA, located 20 bp upstream of the major polyadenylation site at position 7505 (Figs. 5 and 10). The minor rat IL6 mRNA species contains an additional 1203 nucleotides in its 3′-nontranslated region and is generated by using the canonical polyadenylation signal AATAAA at position 8692 and the polyadenylation site at position 8708 (Figs. 5 and 10). Two potential polyadenylation signals and polyadenylation sites have also been detected by sequence analysis of variant human IL6 cDNA clones. While the clones published by Yasukawa and colleagues (49) are generated by using the site corresponding to the minor site in the rat gene (Fig. 10), clones generated by utilization of the more 5′-located site, corresponding to the major polyadenylation site of the rat gene, have also been found.[5] The published cDNA clones of murine IL6 utilize the more 3′-located polyadenylation site (Fig. 10). However, at the same distance as in the human gene, 78 bp upstream of this site, the same polyadenylation signal AATGAA is found in the murine and rat IL6 genes. We do not know whether this signal is also used for the generation of a second murine IL6 mRNA species. It is also unknown whether the mRNA species generated by different polyadenylation have different structural or functional properties (mRNA folding, mRNA half-life, transport, translation efficiency, etc.).

With the availability of the rat IL6 genomic clones, the 2.9 kb of 5′-flanking region sequence and a novel rat macrophage-like cell line with inducible expression of the IL6 gene, the tools are now in hand for a further investigation of the signals and molecular mechanisms leading to the induction of this gene. This induction is a key event in the early stages of an inflammatory response and is possibly also involved in the process of growth transformation.

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REFERENCES

1. Kishimoto, T., and Hirano, T. (1988) *Annu. Rev. Immunol.* **6**, 485–512
2. Hirano, T., Yasujawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyama, K., Iwamatsu, K., Tasunasawa, S., Sakiyama, F., Matsui, H., Takahara, Y., Taniguchi, T., and Kishimoto, T. (1986) *Nature* **324**, 73–76
3. Van Damme, J., Opdenakker, G., Simpson, R. J., Rubira, M. R., Cayphas, S., Vink, A., Billiau, A., and Van Snick, J. (1987) *J. Exp. Med.* **165**, 914–919
4. Nordan, P. R., and Potter, M. (1986) *Science* **233**, 566–569
5. Garman, R. D., Jacobs, K. A., Clark, S. C., and Raulet, D. H. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 7629–7633
6. Takai, Y., Wong, G. G., Clark, S. C., Burakoff, S. J., and Herrmann, S. H. (1988) *J. Immunol.* **140**, 508–512
7. Nakamura, T., Arakaki, R., and Ichihara, A. (1987) *Exp. Cell. Res.* **179**, 488–497
8. Ikebuchi, K., Wong, G. G., Clark, S. C., Ihle, J. N., Hirai, Y., and Ogawa, M. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 9035–9039
9. Wong, G. G., and Clark, S. C. (1988) *Immunol. Today.* **9**(5), 137–139
10. Gauldie, J., Richards, C., Harnish, D., Lansdorp, P., and Baumann, H. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 7251–7255
11. Fey, G. H., and Gauldie, J. (1989) in *Progress in Liver Disease Vol. 9*, (Popper, H., and Schaffner, F., eds) W. B. Saunders, Philadelphia, in press
12. Helle, M., Brakenhoff, J. P. J., de Groot, E. R., and Aarden, L. A. (1988) *Eur. J. Immunol.* **18**, 957–959

13. Baumann, H., Jahreis, G. P., Sauder, D. N., and Koj, A. (1984) *J. Biol. Chem.* **259**, 7331–7342
14. Baumann, H., Hill, R. E., Sauder, D. N., and Jahreis, G. P. (1986) *J. Cell. Biol.* **102**, 370–383
15. Jirik, F. R., Podor, T. J., Hirano, T., Kishimoto, T., Loskutoff, D. J., Carson, D. A., and Lotz, M. (1989) *J. Immunol.* **142**, 144–147
16. Hirano, T., Matsuda, T., Turner, M., Miyasaka, N., Buchan, G., Tang, B., Sato, K., Shimizu, M., Maini, R., Feldmann, M., and Kishimoto, T. (1988) *Eur. J. Immunol.* **18**, 1779–1801
17. Fey, G. H., Hattori, M., Northemann, W., Abraham, L. J., Baumann, M., Braciak, T. A., Fletcher, R. C., Gauldie, J., Lee, F., and Fey, M. F. (1989) *Ann. N. Y. Acad. Sci.* **557**, 317–331
18. Lotz, M., Jirik, F. R., and Carson, D. A. (1989) *Ann. N. Y. Acad. Sci.* **557**, 509–511
19. Tovey, M. G., Content, J., Gresser, I., Gugenheim, J., Blanchard, B., Guymarho, J., Poupart, P., Gigou, M., Shaw, A., and Fiers, W. (1988) *J. Immunol.* **141**, 3106–3110
20. Kohase, M., May, L. T., Tamm, I., Vilcek, J., and Sehgal, P. B. (1987) *Mol. Cell. Biol.* **7**, 273–280
21. VanDamme, J., Cyphas, S., and Opdenakker, G. (1987) *Eur. J. Immunol.* **17**, 1–7
22. Walther, Z., May, L. T., and Sehgal, P. B. (1988) *J. Immunol.* **140**, 974–977
23. Zhang, Y., Lin, J.-X., and Vilček, J. (1988) *J. Biol. Chem.* **263**, 6177–6182
24. Helfgott, D. C., May, L. T., Sthoeger, Z., Tamm, I., and Sehgal, P. B. (1987) *J. Exp. Med.* **166**, 1300–1309
25. Sehgal, P. B., Helfgott, C. D., and Santhanam, U. (1988) *J. Exp. Med.* **167**, 1951–1956
26. Kohase, M., Henriksen-De Stefano, D., Sehgal, P. B., and Vilček, J. (1987) *J. Cell. Physiol.* **132**, 271–278
27. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Turpen, T. H., and Griffith, O. M. (1986) *Biotechniques* **4**, 11–12
29. Gehring, M. R., Shiels, B. R., Northemann, W., de Bruijn, M. H. L., Kan, C.-C., Chain, A. C., Noonan, D. J., and Fey, G. H. (1987) *J. Biol. Chem.* **262**, 446–454
30. Shiels, B. R., Northemann, W., Gehring, M. R., and Fey, G. H. (1987) *J. Biol. Chem.* **262**, 12826–12831
31. Okayama, H., and Berg, P. (1983) *Mol. Cell. Biol.* **3**, 280–289
32. Chiu, C. P., Moulds, C., Coffmann, R. L., Rennick, D., and Lee, F. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7099–7103
33. Van Snick, J., Cayphas, S., Szikora, J. P., Renauld, J. C., Van Roost, Boon, Th., and Simpson, R. J. (1988) *Eur. J. Immunol.* **18**, 193–197
34. Bankier, A. T., Weston, K. M., and Barrell, B. G. (1987) *Methods Enzymol.* **155**, 51–92
35. Staden, R. (1986) *Nucleic Acids Res.* **14**, 217–232
36. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395
37. Hattori, M., and Sakaki, Y. (1986) *Anal. Biochem.* **152**, 232–238
38. Andus, T., Geiger, T., Hirano, T., Northoff, H., Ganter, U., Bauer, J., Kishimoto, T., and Heinrich, P. C. (1987) *FEBS Lett.* **221**, 18–22
39. Northemann, W., Shiels, B. R., Braciak, T. A., Hanson, R. W., Heinrich, P. C., and Fey, G. H. (1988) *Biochemistry* **27**, 9194–9203
40. Tsuchiya, Y., Hattori, M., Hayashida, K., Ishibashi, H., Okubo, H., and Sakaki, Y. (1987) *Gene (Amst.)* **57**, 73–80
41. De Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., and Subramani, S. (1987) *Mol. Cell. Biol.* **7**, 725–737
42. Sargent, T. D., Wu, J. R., Sala-Trepat, J. M., Wallace, R., Reyes, A. A., and Bonner, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 3256–3260
43. VanSnick, J., Cayphas, S., Vink, A., Uyttenhove, C., Coulie, P. G., and Simpson, R. J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 9679–9683
44. Nordan, R. P., Pumphrey, J. G., and Rudikoff, S. (1987) *J. Immunol.* **139**, 813–817
45. Takabe, Y., Seiki, M., Fujisawa, J. I., Hoy, P., Yokota, K., Arai, K. I., Yoshida, M., and Arai, N. (1988) *Mol. Cell. Biol.* **8**, 466–472
46. D'Ambrosio, E., Waitzkin, S. D., Witney, F. R., Salemme, A., and Furano, A. V. (1986) *Mol. Cell. Biol.* **6**, 411–424
47. Gebhard, W., and Zachau, H. G. (1983) *J. Mol. Biol.* **170**, 255–270
48. Hattori, M., Kuhara, S., Takenaka, O., and Sakaki, Y. (1986) *Nature* **321**, 625–628
49. Yasukawa, K., Hirano, T., Watanabe, Y., Muratani, K., Matsuda, T., Nakai, S., and Kishimoto, T. (1987) *EMBO J.* **6**, 2939–2945
50. Tanabe, O., Akira, S., Kamiya, T., Wong, G. G., Hirano, T., and Kishimoto, T. (1988) *J. Immunol.* **141**, 3875–3881
51. Lathey, J. L., and Oldstone, M. B. A. (1988) *Am. J. Pathol.* **132**, 586–592
52. May, L. T., Santhanam, U., Tatter, S. B., Ghrayeb, J., and Sehgal, P. B. (1988) In *Tumor Necrosis Factor/Cachectin: Lymphotoxins and Related Cytokines.* (Bonavida, B., and Kirchner, H., eds) p. 259, S. Karger, Basel

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CLONING OF RAT INTERLEUKIN-13 (IL-13) cDNA AND ANALYSIS OF IL-13 GENE EXPRESSION IN EXPERIMENTAL GLOMERULONEPHRITIS

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SUMMARY Using a reverse transcriptase-polymerase chain reaction method, a cDNA for the complete coding region and part of the 5´-untranslated region of rat IL-13 was cloned from rat renal cortex RNA following the induction of anti-glomerular basement membrane antibody-induced glomerulonephritis. The coding region of IL-13 cDNA displays 74% and 87% sequence identity with the coding regions of human and mouse IL-13 cDNA, respectively. The deduced amino acid sequence of rat IL-13 reveals 63% and 79% homology with the human and mouse proteins, respectively. Using the rat IL-13 cDNA as a molecular probe, increased production of IL-13 mRNA in renal and splenic tissue is demonstrated in the first 48 hours of antibody-induced glomerulonephritis in the rat. The data suggest a role for IL-13 in the inflammatory response *in vivo*.

Interleukin-13 (IL-13) is a recently discovered lymphokine produced by the T_H2 subset of helper T cells (1,2). The genes for human and murine IL-13 have been cloned and characterized (1-4). Human and murine IL-13 proteins display approximately 60% amino acid sequence identity but lack significant homology with other lymphokines (1,2). Recombinant IL-13 stimulates the proliferation of anti-IgM or anti-CD40 activated human B lymphocytes and induces IL-4-independent IgG4 and IgE synthesis (2,5,6). It also induces the differentiation of human monocytes into dendritic-like cells and upregulates the surface expression of MHC class II antigens and the low-affinity receptor for IgE (CD23 or FcεRII) on these cells (2). Like IL-4 and IL-10, IL-13 displays anti-inflammatory actions on macrophages (1). It inhibits IL-1β, IL-6, IL-8, and TNFα mRNA production in lipopolysaccharide-treated monocytes (1).

Anti-glomerular basement membrane (anti-GBM) antibody-induced glomerulonephritis in the rat is an inflammatory renal disease characterized by infiltration of the kidneys with

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Abbreviations: **CD**, cluster of differentiation; **GBM**, glomerular basement membrane; **IFN**, Interferon; **IL**, Interleukin; **RT-PCR**, reverse transcriptase - polymerase chain reaction; T_H, helper T-lymphocyte; **TNF**, Tumor Necrosis Factor; **UDG**, Uracil DNA Glycosylase.

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neutrophils, T-lymphocytes, and macrophages (7). In this study, we report the isolation of a rat IL-13 cDNA clone and its application as a molecular probe to examine IL-13 gene expression during the inflammatory response *in vivo*. We demonstrate an increase in IL-13 mRNA in rat glomeruli between 2 and 48 hours following the induction of glomerulonephritis. Based on its *in vitro* actions on macrophages, IL-13 may play a modulatory role in inflammatory renal disease.

MATERIALS AND METHODS

Induction of Glomerulonephritis. Male Sprague-Dawley rats (200-250 gms) (Charles River, MA) were injected in the femoral vein with 150 μl of rabbit anti-rat GBM serum (kind gift of George F. Schreiner, CV Therapeutics, Palo Alto, CA). Control animals were injected with an equal volume of normal rabbit serum. Rats were sacrificed at 2, 16, 24 and 48 hours post-injection (n=4 per time point). Kidneys were flushed with ice-cold Hanks Balanced Salt Solution (HBSS) until blanched and removed surgically. Glomeruli were isolated on ice by graded sieving (8) and used immediately for RNA extraction. Disease induction was confirmed by histological examination of the renal tissue and by measuring urinary protein to creatinine ratio at time of sacrifice.

Isolation of Rat IL-13 cDNA Clones. A RT-PCR cloning approach was utilized to isolate a cDNA encoding rat IL-13. Total cellular RNA was extracted from the cortex of glomerulonephritic kidneys by the Chomczynski method using RNAzol B reagent (Biotecx Laboratories, TX) (9). RNA was reverse transcribed using an oligo-dT primer and Moloney Murine Leukemia Virus reverse transcriptase (Perkin Elmer, CT). Two rounds of PCR amplification were performed using nested pairs of oligonucleotide primers (Table I). Both primer pairs were selected based on areas of high homology in the untranslated regions (UTR) of human and mouse IL-13 cDNA (1,4). Two μg of reverse transcribed RNA was amplified under the following conditions: 10mM Tris HCl (pH8.3), 50mM KCl, 1mM $MgCl_2$, 5% Dimethyl Sulfoxide (DMSO) (Sigma, MO), 0.2mM of each dNTP, 500 nM of each primer (pair A), 2.5 units of *Thermus aquaticus* DNA polymerase (Taq polymerase, Perkin-Elmer, CT), denaturation at 94°C for 1 min, annealing at 42°C for 2 min, extension at 72°C for 2 min for 40 cycles followed by extension at 72°C for 5 minutes using a DNA Thermal Cycler 480

TABLE I. PCR PRIMERS UTILIZED IN THE ISOLATION OF RAT IL-13 cDNA

Primer pair 1:	*Sense*	5´-gacaagccagcagccta-3´
	Anti-sense	5´-ggcaggcagtgcaggctgagg-3´
Primer pair 2:* (nested)	*Sense*	5´-tggctctgggcttcatggc-3´
	Anti-sense	5´-tgtgagaaaggaaaatga-3´

*In order to clone the RT-PCR product into pAMP1, both primers were designed to include deoxy-UMP residues at the 5´ end as follows: 5´-caucaucaucau-3´.

(Perkin-Elmer, CT) . 5μl of the above PCR product (total volume = 100μl) were removed and re-amplified under the same conditions using nested primer pair B (Table I & Figure 1). Amplified products were electrophoresed in 3% SeaKem LE agarose (American Bioanalytical, MA). Expected target DNA (~470 bp) was excised from the gel and purified by phenol/chloroform extraction and ethanol precipitation according to standard techniques (10) and cloned into pAMP1 plasmid utilizing Uracil DNA Glycosylase according to instructions provided with the CloneAmp^R System (Gibco BRL, MD)(11). Following transformation of *E. coli* DH5α (BRL, MD), separate colonies were picked, grown overnight, and plasmid DNA purified (Qiagen, CA). Sequencing of both strands of the cloned PCR product was performed by the Sanger method (12) using oligonucleotide primers that anneal to the SP6 and T7 promoter regions of pAMP1 (Gibco BRL, MD).

Analysis of IL-13 Gene Expression. Based on the sequence of the isolated rat IL-13 cDNA, a species specific PCR primer pair which amplifies a 279 bp segment of rat IL-13 was designed using PCRPLAN software (*pcgene*, Switzerland). The sequences for the sense and antisense primers were 5'-cagggagcttatcgaggagc-3´ and 5'-aagttgcttggagtaattgagc-3´, respectively. Two μg of total RNA extracted from glomeruli at 0, 2, 16, 24, and 48 hours following the induction of glomerulonephritis was reverse transcribed as described above and PCR amplified under the following conditions: 10mM Tris.HCl (pH8.3), 50mM KCl, 0.6mM $MgCl_2$, 5% DMSO, denaturation at 94°C for 1 min, annealing at 55°C for 45 sec, extension at 72°C for 45 sec for 40 cycles. PCR products were electrophoresed on 2% agarose gels.

Southern Blotting. PCR product electrophoresed as described above was transferred to Hybond-N+ nylon membrane (Amersham, IL) by capillary Southern blotting (10) and covalently linked by UV-irradiation using a Stratalinker UV crosslinker (Stratagene, CA). Rat IL-13 cDNA was purified from the plasmid pAMP1-IL13 following digestion with EcoRI and BamH1 (New England Biolabs, MA). The cDNA was denatured and labeled with the enzyme horseradish peroxidase using the Enhanced Chemiluminescence (ECL) direct nucleic acid labelling and detection system (Amersham, USA). The membrane was hybridized overnight with the labeled cDNA probe in 0.5M NaCl solution at 42°C. Following hybridization, the membrane was washed in 0.5xSSC and detection reagents were added as described by the manufacturer (Amersham, IL). Autoradiography was performed at room temperature for 1 minute.

RESULTS AND DISCUSSION

Rat IL-13 cDNA cloning. The RT-PCR cloning strategy utilized in this study resulted in the isolation of a single rat IL-13 cDNA (Fig. 1). The nucleic acid sequence of the RT-PCR product cloned into pAMP1 was identical in six different clones that were sequenced. The longest open reading frame encodes a 131 amino acid polypeptide and displays 74% and 87% nucleic acid sequence identity with the coding regions of hIL-13 and mIL-13, respectively. Deduced amino acid sequence of rat IL-13 reveals 63% and 79% homology with the human and mouse proteins, respectively (Fig. 2). The probable signal peptide cleavage is predicted to occur after amino acid 20 resulting in a mature polypeptide of 111 amino acids. The predicted molecular weight of the mature protein is 12,126 Da. All five cysteine residues, including one

```
                10        20        30        40        50
                |         |         |         |         |
  1 TGGCTCTGGGCTTCATGGCACTCTGGGTGACTGCAGTCCTGGCTCTCGCT
 51 TGCCTTGGTGGTCTTGCCACCCCAGGGCCAGTGCGGAGATCCACATCTCC
101 CCCTGTGGCCCTCAGGGAGCTTATCGAGGAGCTGAGCAACATCACACAAG
151 ACCAGAAGACTTCCCTGTGCAACAGCAGCATGGTATGGAGCGTGGACCTG
201 ACAGCTGGCGGGTTCTGTGCAGCCCTGGAATCCCTGACCAACATCTCCAG
251 TTGCAATGCCATCCACAGGACCCAGAGGATATTGAATGGCCTCTGTAACC
301 AAAAGGCCTCGGATGTGGCTTCCAGCCCCCCAGATACCAAAATCGAAGTA
351 GCCCAGTTTATATCAAAACTGCTCAATTACTCCAAGCAACTTTTCCGCTA
401 TGGCCACTGAGGGGAGACCGGCCCTGGACATCTCAGCTGTGGACCTCAGT
451 TGTGGATTCATTTTCCTTTCTCACA
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FIGURE 1. Nucleotide sequence of rat IL-13 cDNA. The initiation and stop codons of the longest open reading frame are double underlined. Underlined nucleotides correspond to mouse oligonucleotide primers used to amplify the rat IL-13 cDNA (primer pair 2, Table I).

in the putative signal sequence, are conserved in human, rat, and mouse IL-13. Rat IL-13 contains four potential N-linked glycosylation sites, three of which are conserved in human and mouse IL-13. Rat IL-13 does not share any significant amino acid identity with rat IL-4 (13).

The cross-species RT-PCR cloning strategy utilized in this study allows high efficiency isolation of target cDNA. Performing a second round of PCR using a nested primer pair made possible the cloning of rat IL-13 cDNA from non-lymphatic tissue which contains relatively few

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MOUSEIL13 - MALWVTAVLALACLGGLAAPGPVPRSVSLPLTLKELIEELSNITQDQ-TP -49
RATIL13   - MALWVTAVLALACLGGLATPGPVRRSTSPPVALRELIEELSNITQDQKTS -50
HUMANIL13 - MALLLTTVIALTCLGGFASPGPVPPST----ALRELIEELVNITQNQKAP -46

MOUSEIL13 - LCNGSMVWSVDLAAGGFCVALDSLTNISNCNAIYRTQRILHGLCNRKAPT -99
RATIL13   - LCNSSMVWSVDLTAGGFCAALESLTNISSCNAIHRTQRILNGLCNQKASD -100
HUMANIL13 - LCNGSMVWSINLTAGMYCAALESLINVSGCSAIEKTQRMLSGFCPHKVSA -96

MOUSEIL13 - TVSSLP---DTKIEVAHFITKLLSYTKQLFRHGPF  -131
RATIL13   - VASSPP---DTKIEVAQFISKLLNYSKQLFRYGH   -131
HUMANIL13 - GQFSSLHVRDTKIEVAQFVKDLLLHLKKLFREGRFN -132
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FIGURE 2. Homology alignment of the deduced amino acid sequences of mouse, rat, and human IL-13 proteins. Similarities between two or three species are indicated (|).

lymphocytes. In fact, we isolated rat IL-13 cDNA from renal cortical tissue following the induction of glomerulonephritis during which only a small number of lymphocytes infiltrate the kidney (7).

Analysis of IL-13 gene expression *in vivo*. Injection of antibodies directed to the GBM of the rat kidney induces an inflammatory response characterized by infiltration of the glomerulus by neutrophils, T-lymphocytes, and monocytes (7). The *in vitro* actions of IL-13 on macrophages suggest that this T-lymphocyte-derived lymphokine may modulate macrophage function *in vivo*. In order to determine whether IL-13 is expressed during the course of inflammatory disease, we isolated rat glomeruli following the induction of anti-GBM glomerulonephritis and studied IL-13 mRNA production by RT-PCR. The primer pair utilized is species specific and is based on the rat IL-13 cDNA sequence (Figure 1). As shown in Figure 3A, IL-13 mRNA increases significantly 2 hours following the induction of glomerulonephritis and can still be detected at 48 hours. Control rats injected with normal rabbit serum did not reveal increased glomerular expression of IL-13 over baseline, and GAPDH mRNA expression analysed by RT-PCR was constant over time (data not shown). IL-13 mRNA is also detected in rat spleen following injection of anti-GBM antibody (Fig. 3B). The identity of the PCR product was confirmed by Southern blotting and probing with rat IL-13 cDNA (Fig. 3C).

The data demonstrates expression of the IL-13 gene during an antibody-mediated inflammatory disease. We have previously observed transient T_H2-lymphokine (IL-4 and IL-10) gene expression in rat glomeruli in the first 24 hours of anti-GBM nephritis (14). As predicted

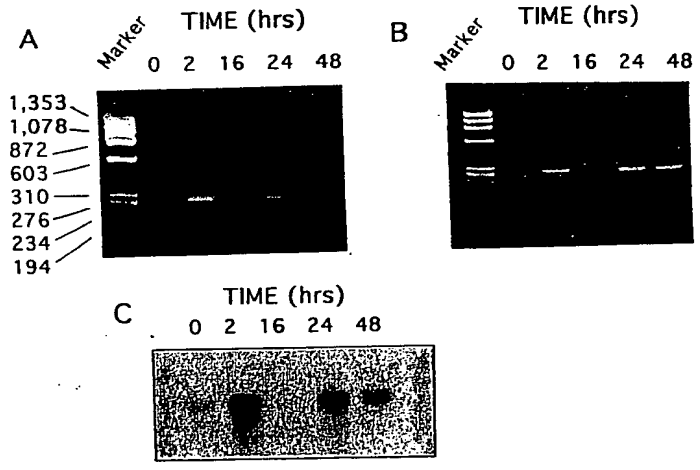

FIGURE 3. Analysis of rat IL-13 mRNA production in rat glomeruli (A) and spleen (B) following the induction of anti-GBM glomerulonephritis. 20μl of RT-PCR product from each time point was subjected to 2% agarose gel electrophoresis and stained with ethidium bromide. Marker: φX174-RF-DNA/Hae-III-fragments, 0.5μg/lane. (C) Southern blot analysis of glomerular RT-PCR product using rat IL-13 cDNA as a chemiluminescent probe.

from the *in vitro* actions of IL-4 and IL-10 (15), this T_H2 response was associated with decreased IL-1 and T_H1-lymphokine (IFNγ and IL-2) mRNA production suggesting that T_H2 lymphokines may modulate glomerular inflammation. Whether IL-13 plays a role in regulating macrophage function *in vivo* remains to be determined. The availability of rat IL-13 cDNA should allow the generation of recombinant rat IL-13 and of neutralizing antibodies necessary for further analysis of the role of IL-13 in experimental glomerulonephritis in the rat.

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REFERENCES

1. Minty, A., Chalon, P., Derocq, J.M., Dumont, X., Guillemont, J.C., Kaghad, M., Labit, C., Leplatois, P., Liauzun, P., Miloux, B., Minty, C., Casellas, P., Loison, G., Lupker, J., Shire, D., Ferrara, P., and Caput, D. (1993). Nature 362:248-250.

2. McKenzie, A.N.J., Culpepper, J.A., De Waal Malefyt, R., Briere, F., Punnonen, J., Aversa, G., Sato, A., Dang, W., Cocks, B.G., Menon, S., De Vries, J.E., Banchereau, J., and Zurawski, G. (1993). Proc. Natl. Acad. Sci. USA 90:3735-3739.

3. Brown, K.D., Zurawski, S.M., Mosmann, T.R., and Zurawski, G. (1989). J. Immunol. 42:679-687.

4. McKenzie, A.N.J., Li, X., Largaespada, D.A., Sato, A., Kaneda, A., Zurawski, S.M., Doyle, E.L., Milatovich, A., Francke, U., Copeland, N.G., Jenkins, N.A., and Zurawski, G. (1993). J. Immunol. 150:5436-5444.

5. Punnonen, J., Aversa, G., Cocks, B.G., McKenzie, A.N.J., Menon, S., Zurawski, G., De Waal Malefyt, R., and De Vries, J.E. (1993). Proc. Natl. Acad. Sci. USA 90:3730-3734.

6. Cocks, B.G., De Waal Malefyt, R., Galizzi, J.P., De Vries, J.E., and Aversa, G. (1993). Int. Immunol. 5:657-663.

7. Tipping, P. G., Neale, T.J., and Holdsworth, S.R. (1985). Kidney Int. 27:530-537.

8. Fujiwara, Y., Kiamura, E., Ueda, N., Fukunaga, M., Orita, Y., and Kamada, T. (1989). Kidney Int. 36:985-991.

9. Chomczynski, P., and Sacchi, N. (1987). Anal. Biochem. 162:156-159.

10. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds), (1990). Current Protocols in Molecular Biology, Vol. 1. Wiley Interscience, New York.

11. Nisson, P.E., Rashtchian, A., and Watkins, P.C. (1991). PCR Meth. and Appl. 1:120-123.

12. Sanger, F., Nicklen, S., and Coulson, A.R. (1977). Proc. Natl. Acad. Sci. USA 74:5463-5467.

13. McKnight, A.J., Barclay, A.N., and Mosen, D.W. (1991). Eur. J. Immunol. 138:1187-1194.

14. Lakkis, F.G., Cruet, E.N., Fukunaga, M., and Munger, K.A. (1993). Journal of the American Society of Nephrology 4:613 *(abstract)*.

15. Moore, K.W., O'Garra, A., De Waal Malefyt, R., Vieira, P., and Mosmann, T.R. (1993). In Annual Review of Immunology (W.E. Paul, C.G. Fathman, and H. Metzger, Eds.). pp.165-190. Annual Reviews Inc., Palo Alto, CA.

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SYNTHESIS AND CHARACTERIZATION OF RAT INTERLEUKIN-10 (IL-10) cDNA* CLONES FROM THE RNA OF CULTURED OX8$^-$ OX22$^-$ THORACIC DUCT T CELLS

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SUMMARY A cDNA of the complete coding region of rat IL-10 was cloned and sequenced using RNA isolated from a cultured population of thoracic duct T-lymphocytes obtained from *Trichinella spiralis* infected animals. The OX8$^-$ OX22$^-$ T-helper cells were stimulated *in vitro* with Concanavalin A for 24 hours prior to harvest. Reverse transcription of cellular RNA was primed with oligo-dT followed by amplification of IL-10 specific cDNA by polymerase chain reaction with synthetic oligo nucleotide primers chosen from two highly conserved regions of mouse and human IL-10. The sequence of the coding region of the amplified, cloned rat IL-10 cDNA is 90% identical to the mouse and 82% identical to the human IL-10 cDNA coding regions.

Mouse interleukin 10 (mIL-10) was originally named cytokine synthesis inhibitory factor (CSIF) due to the inhibitory activity of this molecule on the synthesis of interferon gamma (IFN-γ) by a mouse T cell clone (1). Analysis of the cDNA and protein sequences of mIL-10 indicated striking homology with a partially characterized product, BCRFI, encoded by the Epstein-Barr virus genome (2). A human cDNA of IL-10 (hIL-10) was characterized and shown to be nearly 73% identical in deduced amino acid sequence with the deduced amino acid sequence of mIL-10. Both hIL-10 and BCRFI have been shown to inhibit IFN-γ production (3). Murine IL-10 has been shown to stimulate proliferation of CD4$^-$ CD8$^-$ thymocytes (4). Human monocytes, in response to LPS stimulation, produce IL-10 which may function in an autoregulatory role by down regulating class II MHC molecules and inhibiting the production of proinflammatory cytokines (5). During parasitic infections the establishment of resistance or

*Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. L02926.

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Abbreviations: BCRFI, Epstein-Barr virus open reading frame I; CSIF, cytokine synthesis inhibitory factor; Con A, concanavalin A; IL-, interleukin-; RT-PCR, reverse transcriptase-polymerase chain reaction.

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acute disease appears to be at least partly determined by the dominance of IL-10 or IFN-γ, depending on the specific parasite (6,7).

Our preliminary studies examining the cytokine profile of a protective T helper cell population ($OX8^-$ $OX22^-$) isolated from the thoracic duct of rats infected with the nematode *Trichinella spiralis* (8), indicated that these cells exhibit some characteristics of Th2 phenotype (9). Stimulation of the isolated cell population in vitro with Con A or *T. spiralis* specific antigen, led to the secretion of factors that caused isotype switching of B cells from IgM to IgE, an IL-4 response, and the differentiation of eosinophils from bone marrow cells, an IL-5 response (unpublished, K. Ramaswamy and R. Bell). Analysis of RNA from these cells by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers for IL-2, IL-4, IL-5 and IFN-γ indicated that mRNA of all four cytokines were present in the population (unpublished, R.Goodman). Identical analyses of the non-protective T cell population ($OX8^-$ $OX22^+$) which was isolated concurrently from the same animals and stimulated identically, produced a similar mRNA profile but those cells were unable to elicit eosinophil differention even though IL-5 mRNA was present. Both cell populations contain significant levels of IFN-γ mRNA. To determine whether the differences between these cell populations where in part due to differences in IFN-γ or IL-10 production, we have developed a RT-PCR assay for rat IL-10 using an oligonucleotide primer identical to the translation initiation coding region of mIL-10, having three mismatches with human IL-10, and an antisense oligonucleotide for the 3'-untranslated region which is complementary to mIL-10, but differs in the distal half from hIL-10. To verify the identity of the product we have cloned and sequenced the resulting cDNA and report our findings here.

MATERIALS AND METHODS

Cellular RNA was isolated from pulverized frozen rat tissues and from separated Con A stimulated $OX8^-$ $OX22^-$ thoracic duct T lymphocytes (8) of *T. spiralis* infected AO rats, by the guanidine/acidic phenol:chloroform method of Xie and Rothblum (10). RNA concentrations were determined by measuring UV absorbance at 260 nm. The integrity of the RNA was checked by visualizing the ethidium bromide stained rRNA bands in the formaldehyde denaturing gel used for the Northern blot which was performed as described earlier (11).

Oligonucleotides for IL-10 were selected by comparing mouse (1,13) and human (3) sequences to identify highly conserved regions. Specific sequences of the oligos are identical to those of mouse IL-10 at the translation initiation site in exon 1 (oligo#1, 5'-d[catgcctggctcagcactgc], sense strand), in the 3'-untranslated region (oligo#2, 5'-d[gggaactgaggtatcagagg], antisense strand), and in mid-exon 1 (oligo#3, 5'-d[gactgggaagtgggtgcag], antisense strand). Oligo#1 and oligo#2 were used as primers for polymerase chain reaction (PCR) amplification. Oligo#3 was labeled and used to probe Southern blots.

RT-PCR reactions were performed to clone rat IL-10 and to detect small quantities of IL-10 mRNA using cellular RNA, oligo#1 and oligo#2 (above) and a GeneAmp RNA PCR Kit (Perkin-Elmer) following the manufacturers directions. To clone the rat IL-10 cDNA, total RNA was used from $OX8^-$ $OX22^-$ cells (above) which were stimulated in vitro with Con A for 24 hours before collection. First strand synthesis of 0.5 μg of RNA was performed at 42^oC using Oligo-dT as the primer. PCR was accomplished by the addition of AmpliTaq DNA Polymerase and oligonucleotide primers #1 and #2 (to a 250 nM final concentration of each),

followed by 35 cycles of amplification (94°C, 45 s; 50°C, 45 s; and 72°C, 80 s) and a final primer extension at 72°C for 5 minutes. Comparative IL-10 RT-PCR assays using cellular RNA from various tissues were performed in an identical fashion except the PCR was stopped after 25 cycles and identical samples of 0.03 ug of RNA were also analyzed. Southern blotting for detection of the amplified IL-10 cDNA products was accomplished electrophoresis of samples in a 3% NuSieve 3:1 agarose gel (FMC) with 1 X Tris-acetate-EDTA buffer (15), denaturation of the DNA with sodium hydroxide and neutralization with Tris base (pH 8) followed by capillary transfer to GeneScreen Plus membranes (DuPont-New England Nuclear) with 10 X SSC (12). Blots were prehybridized and hybridized in the same solution (20 mM Tris, pH 8; 6 X SSPE ; 1 mM EDTA; 5 X Denhardt's solution; 50 μg/ml sheared, denatured Salmon sperm DNA; and 0.5% SDS) at 50°C. Oligo#3 was labeled with [^{32}P]cordycepin-5'-triphosphate (DuPont-NEN) using Terminal deoxynucleotide transferase (Promega) as described by Tu and Cohen (14) for use as a probe. Hybridization was at 50°C overnight. Unbound probe was washed off with two incubations each in 6 X SSPE, 0.1% SDS at room temperature and at 50°C. Bands were detected by exposing X-OMAT AR film (Kodak) with washed membranes. Band sizes were determined by comparing migration distances with bands from a 123 bp sizing ladder (Gibco-BRL) electrophoresed beside RT-PCR samples and detected by ethidium bromide staining.

To clone the IL-10 cDNA, a sample from RT-PCR was electrophoresed through 1.0% SeaKem GTG agarose (FMC), with 1 X TAE, then excized from the gel after ethidium bromide staining, and purified with Geneclean (BIO 101). The cDNA, approximately 680 bp long, was ligated with pT7Blue(U) plasmid DNA (Novagen, Inc.) using T4 DNA Ligase (New England Biolabs). NovaBlue competent *Escherichia coli* were transformed with ligation products and recombinant colonies were selected on Ampicillin/Tetracycline plates containing color substrates for the Lac Z gene product. White colonies were screened by hybridization with ^{32}P-labeled oligo#3 to select IL-10 bearing plasmids which were amplified and purified from "minipreps" (15).

Plasmids were denatured with sodium hydroxide, and sequenced with Sequenase 2.0 (U.S. Biochemicals) using vector specific primers T7 5'-d(taatacgactactataggg) and M13 Forward-40 5'-d(gttttcccagtcacgac), with separation by electrophoresis through a 5% HydroLink Long Ranger gel (J.T.Baker) and as described earlier (11). Sequencing reaction products were detected by autoradiography and were compared with mouse and human IL-10 sequences using the ALIGN program (Scientific and Educational Software, State Line, PA). Protein structure calculations for the deduced mature protein were accomplished using the IBI Pustell program (Kodak, New Haven, CT).

A Northern blot of cellular RNA from rat brain and Con A stimulated T cells was performed as described earlier (11). The cloned rat IL-10 cDNA insert was labeled with [^{32}P]dATP by random primed cDNA synthesis using a Prime-a-Gene kit (Promega) for use as a probe. The IL-10 mRNA size was determined by comparing the migration distance of the detected band with the distances of 28S and 18S rRNA bands detected by ethidium bromide staining of the original gel.

RESULTS AND DISCUSSION

Reverse transcription and PCR amplification of RNA from Con A stimulated OX8$^-$ OX22$^-$ T cells from *T. spiralis* infected rats, using the mouse IL-10 primers, produced a 682 bp cDNA which hybridized specifically to the internal oligo#3 of mouse IL-10. Ligation of this fragment into a pT7Blue plasmid followed by transformation into *E. coli* produced five clones with approximately 680 bp inserts which were partially sequenced to determine the orientation of the clones within the plasmids. Two clones (pIL10-30 and pIL10-39) which had been inserted in the opposite orientation relative to the multiple cloning site were sequenced completely using

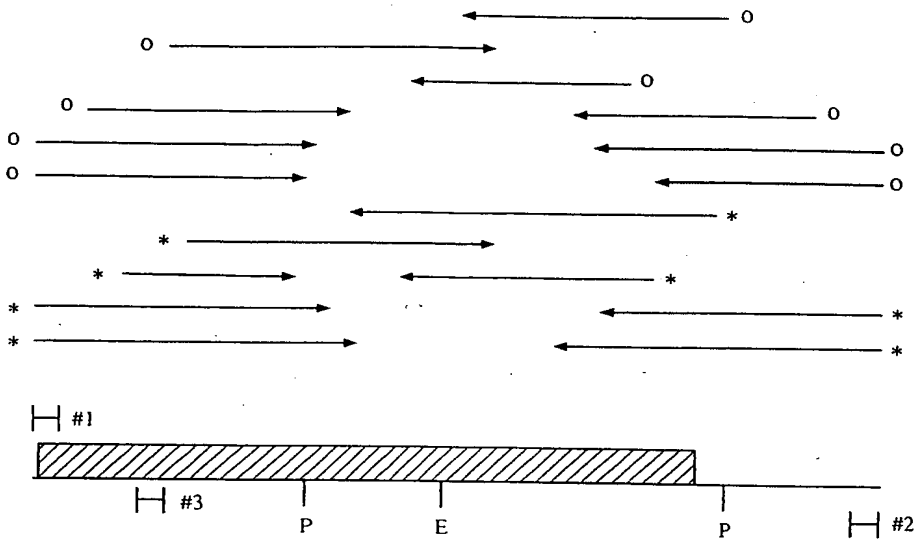

Figure 1. Diagram of the cDNA clone, cDNA sequencing strategy, restriction enzyme sites and deduced protein coding region for rat IL-10. The full sequence of 682 bases includes a single open reading frame (hatched) with a translation initiation codon at base 2 and a termination codon at base 538. The first and last 20 bases of the cDNA represent mouse IL-10. There are two Pst I (P) and one Eco RI (E) restriction sites in the cDNA. The positions of the three oligonucleotides are indicated. Sequence reactions were in the direction indicated by the arrows and represent reactions from pIL10-30 (o), which is oriented with the 5'-end at the T7 end of the multiple cloning site, and pIL10-39 (*), in which the cDNA is oriented oppositely, with the 5'-end near the universal-40 primer site.

replicate sequencing reactions of each strand, with multiple gel loadings to produce a continuous sequence as indicated in Figure 1. The sequence determined for these two clones contained a single open reading frame of 537 bases beginning at base 2, followed by 144 bases of the 3'-untranslated region (rIL-10 in Fig. 2). Other than orientation, the only difference between the two clones is that pIL10-39 is missing the "T" overhang that should exist at the ligation site adjacent to the 5'-end of the cDNA.

Comparison of the resulting open reading frame of this sequence with the coding region of mouse and human IL-10 sequences (1,3) indicated that it is 90% identical to the mouse and 82% identical to human coding region sequences (Fig. 2). However, the first and last 20 nucleotides of our cDNA actually represent mouse IL-10 and could differ slightly from the actual rat sequence. Comparison of the three species indicates unusually strict conservation of the 3' untranslated region in the IL-10 gene. As suggested by Moore et al. (1), there are a number of "AT" rich regions in the 3'-end of mIL-10 which may be involved in regulating mRNA stability and the most proximal of these is conserved in both sequence and position in rat IL-10 (Fig. 2, bases 654-658). In addition, significant regions of homology exist between the 3'-untranslated regions of mIL-10 and hIL-10 beyond the extent of the rIL-10 cDNA (not shown), which include the "AUUUA" motif involved in regulated degradation of some mRNA's (16), suggesting that there is post-transcriptional regulation of IL-10 mRNA.

The deduced amino acid sequence (Fig. 3) should be full length at 178 amino acids, based on the mouse and human sequences. The probable signal peptide cleavage site was predicted

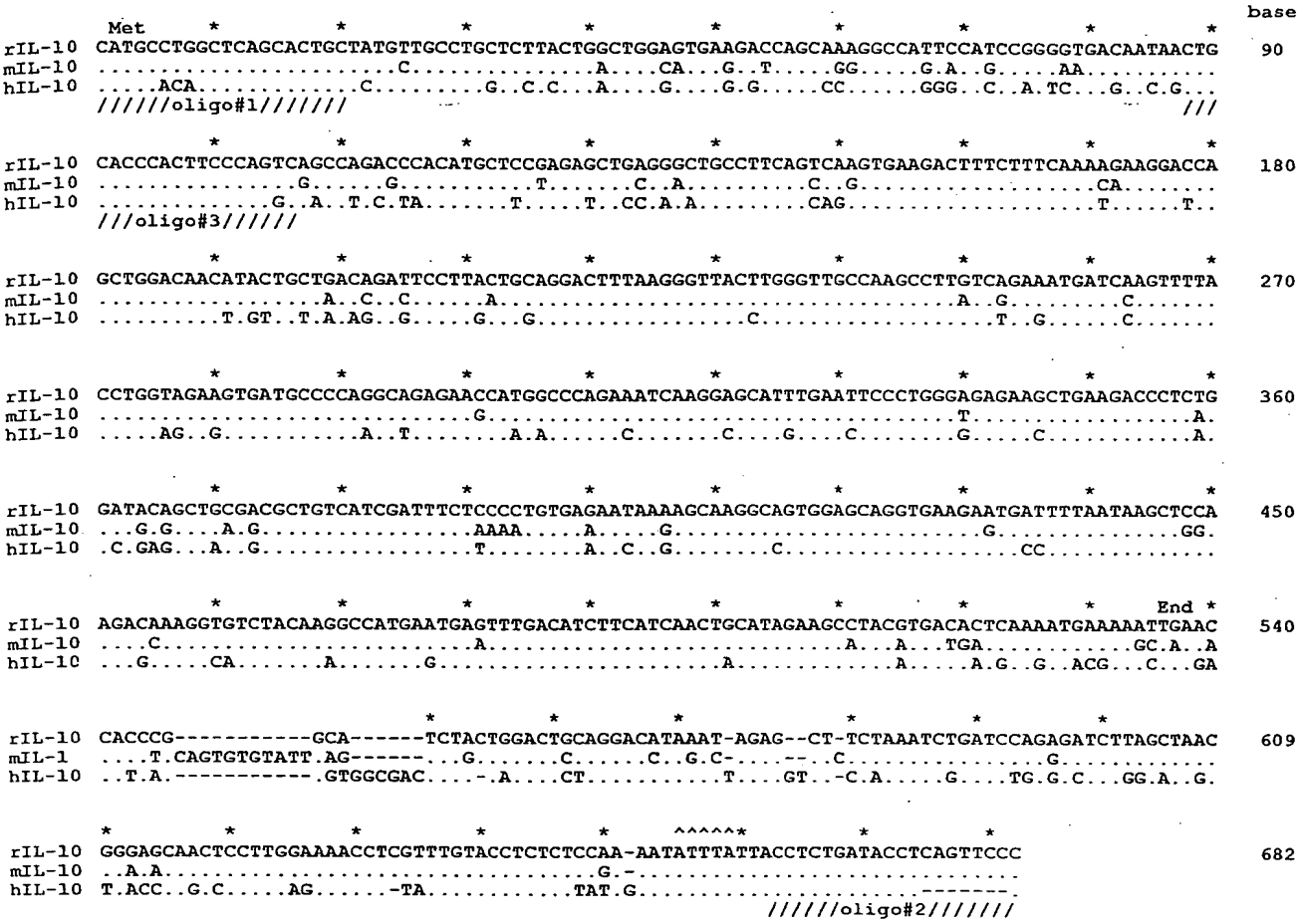

Figure 2. Nucleotide sequence of rat IL-10 cDNA and comparison with mouse and human IL-10 cDNAs. The complete nucleotide sequence of the rat IL-10 cDNA is shown, with the similarities (.) and differences (base code) and gaps (-) in alignment of the mouse and human cDNA sequences are indicated for the corresponding region of the cDNAs. The start codon (Met) and termination (End) are shown. Oligonucleotide positions (hatched) are indicated for the PCR primers (#1 and #2) and for the label used in Southern blots (#3). A putative destabilization signal is marked (^^^^^).

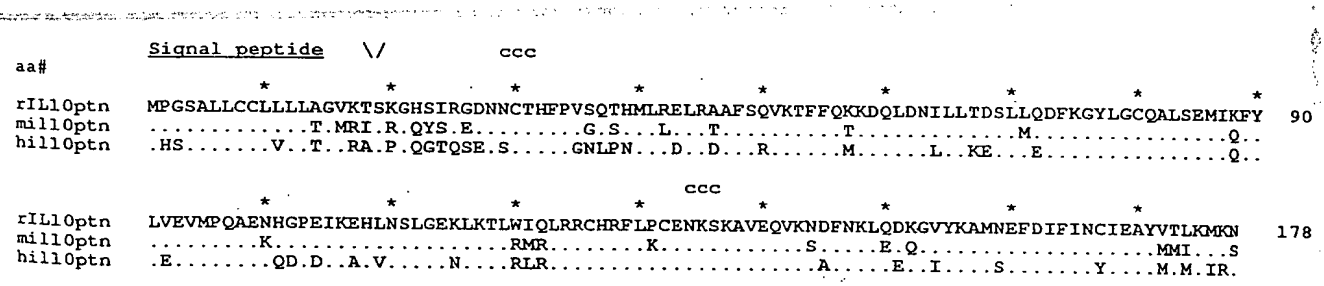

Figure 3. Homology alignment of the deduced amino acid sequences of rat, mouse and human IL-10 peptides. The full-length deduced amino acid sequence of rat IL-10 is shown with the similarities in the mouse and human proteins indicated (.) as well as the differences. The most likely signal sequence clevage site is indicated (\/) after amino acid 18, as are the potential N-linked glycosylation sites of mouse and rat IL-10 (ccc).

based on frequency calculations (17) to occur after amino acid 18, leaving a mature size of 160 amino acids. The predicted molecular weight for the mature protein is 18.6 kDa however, there are two potential N-linked glycosylation sites (Fig. 3) which, if utilized in the native protein, would alter the molecular weight. The first potential N-glycosylation site is present in the rat and mouse but not human deduced protein sequences, whereas the second site is present in all three. The calculated pI of the mature rat protein is 9.1, and the calculated pI values for mouse is 8.7 and the human is 8.2. There are four conserved cysteine residues in the mature IL-10 peptide from all three species, and a fifth in rat and mouse, however the native proteins will have to be analyzed to determine if there are any internal or external disulfide bridges. Comparison of the deduced amino acid sequences of the three IL-10s indicates that mouse and rat share 83% identity while human and rat have 73% identity (Fig. 3). Mouse and human IL-10 peptide sequences match at 72% of the amino acid residues.

The intact mRNA of rat IL-10 is approximately 1700 bases as determined by the position of a single detectable hybridization band relative to the positions of 28S and 18S RNA bands in a Northern blot of cellular RNA from Con A stimulated $OX8^-$ $OX22^-$ T cells (20 μg RNA). This size is similar to that of hIL-10 mRNA (3). IL-10 mRNA was not detected by Northern blot analysis of 20 mg total RNA samples from brain, Peyer's patch, upper small intestine and freshly isolated thoracic duct T cells from *T. spiralis* infected rats (not shown).

RT-PCR results analyzing RNA from freshly separated, Con A stimulated and *T. spiralis* antigen stimulated populations of thoracic duct $CD8^+$ and $CD8^-$ T cells indicate that IL-10 mRNA is expressed in the Con A stimulated cells in far greater abundance than the other populations (not shown). Whether the protein is expressed in a similar pattern will be tested when a suitable mAb is prepared.

Acknowledgments: We thank Dr. K. Ramaswamy for isolation and culturing of the $OX8^-$ $OX22^-$ cell population used in this experiment and Anita Hesser for assistance in preparation of this manuscript. This work was supported by NIH grant AI 17484 and Hatch project NY(c)-473400 funds.

REFERENCES

(1) Moore, K.W., Vieira, P., Fiorentino, D.F., Trounstine, M.L., Khan, T.A., and Mosmann, T.R. (1990) Science 248:1230-1234.

(2) Baer, R., Bankier, A.T., Biggin, M.D., Deininger, P.L., Farrell, P,J., Gibson, T.J., Hatfull, G., Hudson, G.S., Satchwell, S.C., Seguin, C., Tuffnell, P.S., and Barrell, B.G. (1984) Nature 310:207-211.

(3) Vieira, P., de Waal-Malefyt, R., Dang, M.-N., Johnson, K.E., Kastelein, R., Fiorentino, D.F., de Vries, J.E., Roncarolo, M.-G., Mosmann, T.R., and Moore, K.W. (1991) Proc. Natl. Acad. Sci. USA 88:1172-1176.

(4) MacNeil, I.A., Suda, T., Moore, K.W., Mosmann, T.R., and Zlotnik, A. (1990) J. Immunol. 145:4167-4173.

(5) de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G., and de Vries, J.E. (1991) J. Exp. Med. 174:1209-1220.

(6) Silva, J.S., Morrissey, P.J., Grabstein, K.H., Mohler, K.M., Anderson, D., and Reed, S.G. (1992) J. Exp. Med. 175:169-174.

(7) Sher, A., Fiorentino, D., Caspar, P., Pearce, E., and Mosmann, T. (1991) J. Immunol. 147:2713-2716.
(8) Bell, R.G., Korenaga, M., and Wang, C.H. (1987) Immunology 61:221-227.
(9) Mosmann, T.R., and Moore, K.W. (1992) Immunol. Today 12:A49-A53.
(10) Xie, W.-Q., and Rothblum, L.I. (1991) BioTechniques 11:325-327.
(11) Goodman, R.E., and Schanbacher, F.L. (1991) Biochem. Biophys. Res. Comm. 180:75-84.
(12) Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
(13) Kim, J.J., Brannan, C.I., Copeland, N.G., Jenkins, N.A., Khan, T.A., and Moore, K.W. (1992) J. Immunol. 148:3618-3623.
(14) Tu, C.-P.D., and Cohen, S.N. (1980) Gene 10:177-83.
(15) Davis, L.G., Dibner, M.D., and Battey, J.E. (1986) Basic methods in molecular biology, Elsevier, New York, NY.
(16) Malter, J.S. (1989) Science 246:664-666.
(17) von Heijne, G. (1986) Nuc. Acids Res. 14:4683-4690.

(9)

Recombinant DNA

SECOND EDITION

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The cover illustration, by Marvin Mattelson, symbolizes some of the elements of this book. The DNA double helix is, of course, central to the book, as it is to the cover illustration. The blocks are double-stranded DNA fragments synthesized by the polymerase chain reaction, a technique that has revolutionized the way molecular genetics experiments are done. The number of fragments doubles repeatedly, going off into the distance (see Chapter 6). The coat colors of the mice running down the helix (in the same direction but with opposite polarity!), are changing from albino to chimeric, then chimeric to agouti. These coat color changes show mice in which genetic engineering has been used to knock out a specific gene. The experiment is shown more realistically in Figure 14-9.

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New Tools for Studying Gene Function

Application of Recombinant DNA to Biotechnology

Impact of Recombinant DNA on Human Genetics

Preface

Application of recombinant DNA techniques to biology is bringing about a revolution in our understanding of living organisms. There is no field of experimental biology that is untouched by the power we now have to isolate, analyze, and manipulate genes. When the first edition of *Recombinant DNA* was published in 1983, recombinant DNA techniques were already being used extensively for the analysis of viral and bacterial genetics, but dissection of eukaryotic genes was only just beginning. There were hints of what was to come. The concept of the gene as a continuous stretch of DNA had been shattered with the discovery of introns, but alternative splicing and genes-within-genes were yet to be revealed. Identification of cellular oncogenes seemed to promise an understanding of cancer, but the mechanisms of their action—and the existence of tumor suppressor genes—were still subjects for speculation. A handful of genetic diseases were being analyzed at the molecular level, but the isolation of the disease genes and the development of gene therapy were yet to come.

Our aim in writing the second edition of *Recombinant DNA* is to show how recombinant DNA techniques have led to the explosion in our knowledge of fundamental biological processes. As in the first edition, which was subtitled *A Short Course*, we provide a concise presentation of the methods, underlying concepts, and far-reaching applications of recombinant DNA technology. The field has grown since the publication of the first edition, and so has our book. But even though our previous subtitle may be inappropriate for this enlarged edition, our approach to the material has remained true to the spirit of the "short course": as before, the uninitiated will find access to the field of recombinant DNA here.

The book is now divided into six major sections. The first five chapters, which are largely unchanged from the first edition, provide a historical introduction to the early development of recombinant DNA technology, up to the point when studies of eukaryotic organisms began in earnest. In the next section we describe in detail the methods currently used to clone and analyze genes, and devote an entire chapter to the polymerase chain reaction, which has had an extraordinary impact on research. The great power of recombinant DNA techniques comes from the ability to explore gene functions by manipulating genes and then introducing them back into cells. The third section of the book discusses how this is done in mammalian cells, yeast, mice, and plants. The fourth section describes the progress these manipulations have allowed in key areas of biology. Here the range of recombinant DNA applications is demonstrated, from the analysis of cell cycle control and embryonic development, to the isolation of genes involved with brain function. Indeed, these techniques have spawned a whole industry—biotechnology. In the fifth section, we describe some of its accomplishments, including the development of genetically engineered pharmaceutical and agricultural products, and the studies of the human immunodeficiency virus that are leading the attack on AIDS. The differences between the first and second editions are perhaps most evident in the final section, where we describe the revolution in human molecular genetics and the ways in which recombinant DNA techniques are providing new methods for diagnosis and treatment of human inherited diseases.

The topics that are covered and the approach we take to describing them make this book suitable for undergraduate and graduate students in molecular biology, cell biology, biochemistry, genetics, or biotechnology courses; for medical students and physicians; and for others who have an interest in recombinant DNA techniques—for example, forensic scientists, patent attorneys, and science journalists.

Textbooks dealing with biochemistry, molecular genetics, and molecular biology usually present information without describing the experiments that were done to obtain it. We think that this is a pity, because designing and doing experiments is exciting and fun. As in the first edition, we have used real experiments to illustrate important biological phenomena, and we have plundered our colleagues' papers for interesting examples. Figures are used profusely to try to make complex real-life experiments intelligible, but inevitably we have not been able to present all the subtle details. Those who want to explore these details will find the experiments in the research papers listed at the end of each chapter, and the review papers we cite will provide an entry point to each topic.

This book is atypical in another regard. Because we do not consider it primarily a textbook for conveying undisputed facts about molecular biology, we have been able to include exciting research that is at the cutting edge of biology. The interpretation of experimental data often changes with time, so the reader should bear in mind that future research might require modification of some of the ideas we present. This is all part and parcel of doing research, because a science that does not change is a dead science. Modern experimental research in biology is an ever-changing dynamic enterprise, and we hope that *Recombinant DNA* conveys the excitement of the continuing process of discovering how organisms work.

Acknowledgments

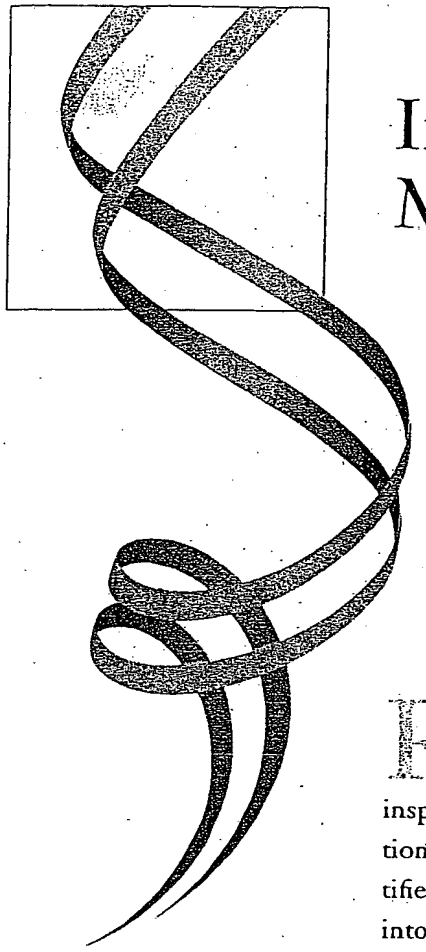
Those who most deserve our thanks are our families, from whom we were taken for many days by this book, and our colleagues at Cold Spring Harbor Laboratory and Genentech, who sometimes had a difficult time communicating with us when we were preoccupied with writing.

We are grateful to the many friends and colleagues who read our manuscript, criticized, corrected, and provided information. They include Sue Alpert, French Anderson, Avi Ashkenazi, David Beach, Martin Bobrow, Tom Caskey, Jeff Chamberlain, Irvin Chen, Francis Collins, Alan Coulson, David Cox, Ken Culver, Kay Davies, Jim Eberwine, Stan Fields, Ted Friedman, Bruce Futcher, Peter Gergen, Richard Gibbs, Paul Godowski, Andre Goffeau, Takashi Gojobori, Kenshi Hayashi, Dan Hartl, Andrew Haitt, Tom Hynes, Paula Jardieu, Karen Johnson, Dan Klessig, Jeff Kuret, Mike Laspia, Philip Leder, Fred Ledley, Vincent Marchesi, Rob Martienssen, Dusty Miller, Rick Myers, Karoly Nikolics, Luis Parada, Scott Putney, Don Rio, David Schlessinger, Matt Scott, John Sulston, Barbara Trask, Rebecca Ward, Robin Weiss, Jim Wells, Ted White, and Bob Williamson. Any errors, nevertheless, are ours, and not theirs.

Elizabeth Zayatz, the development editor, had the unenviable job of trying to make us concentrate on the work at hand when we wanted to be doing other things. She did wonderfully well keeping us at it and became a trusted friend. Bill O'Neal and Jodi Simpson, the manuscript editors, smoothed awkward passages and made us think more carefully about the information we were trying to convey. Janet Tannenbaum, the project editor, combed the manuscript and graphics with a thoroughness that must have required a magnifying glass. She guided us through a forest of edited manuscript, galleys, and page proofs. At times it seemed that never a day went by without an overnight package from Janet arriving on our desks. Alison Lew gave the text, illustrations, and cover their design and Bill Page looked after the art program—tasks that rapidly assumed epic proportions as we all worked to produce the figures that are an essential complement to the text. The figures were rendered by Network Graphics and Tomo Narashima. The beautiful and remarkable cover art is by Marvin Mattelson. Listening to him expound on surrealism and watching him sketch were highlights of the production. Julia De Rosa masterfully coordinated the production process to allow rapid completion of the project. Linda Chaput, President of Scientific American Books, was always patient and understanding. Her guidance and advice were invaluable when the going got tough, and our discussions with her were rewarding, and fun.

James D. Watson Michael Gilman
Jan Witkowski Mark Zoller
December, 1991

CHAPTER



In Vitro Mutagenesis

Recombinant DNA technology and DNA sequencing provided the tools to clone and characterize genes. As we learned in Chapter 8, simple inspection of gene sequences told us much about genomic organization. Functional sequences, such as transcriptional control elements, could often be identified by comparing sequences of a number of genes. However, to delve deeply into the structure and function of genes required the ability to change the DNA sequence and examine the effect of the change on gene function. For decades before the advent of recombinant DNA, this was done by classical genetics, the identification of mutant organisms with new properties. From the genetic properties of mutants, information about the structure and function of the underlying genes could often be inferred. This approach, however, was limited to organisms in which simple genetic analysis was possible—bacteria, yeast, fruit flies. Genetic analysis of more complex, longer-lived organisms like mice and men was slow and difficult.

Recombinant DNA changed all that. The ability to isolate genes as molecular clones, the development of tools to modify gene sequences in the test tube, and the power to return altered genes to the organism to test their function have revolutionized the way genetics is done in higher organisms. Because we now often work "backwards" from gene sequence to gene function, in contrast to

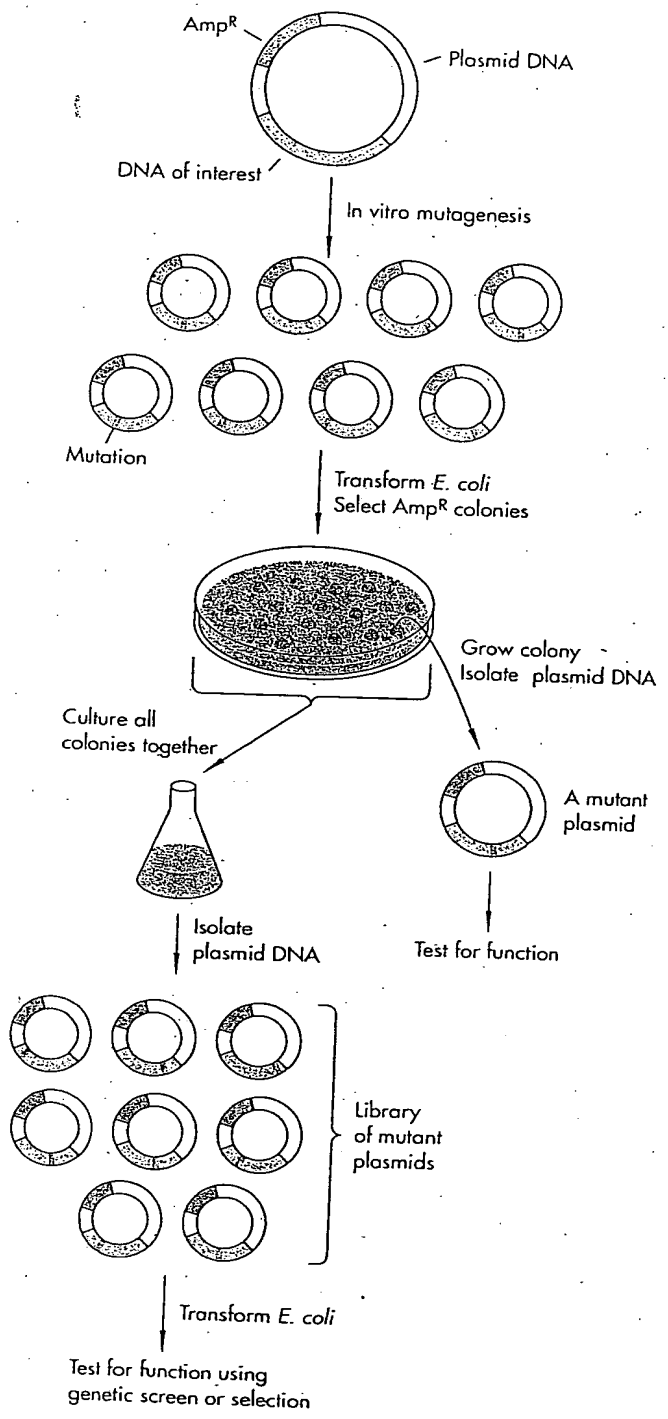


FIGURE 11-1
General strategy for an in vitro mutagenesis experiment. Most procedures for in vitro mutagenesis follow the same basic scheme: Plasmid DNA is "mutagenized" in vitro, then introduced into *E. coli* by transformation. Depending on the method, mutant clones can be isolated and tested individually, or a library of mutant plasmids can be obtained, which are tested using a genetic screen.

classical genetics, this new approach spawned by recombinant DNA is called *reverse genetics*. In this chapter we will learn ways to alter the sequence of a cloned gene at will and how these methods are used to understand the structure and function of genes and gene products.

In Vitro Mutagenesis Is Used to Study Gene Function

In vitro mutagenesis of cloned genes has become a standard tool in the functional analysis of nucleic acids and proteins. Most procedures follow the same basic scheme (Figure 11-1). Plasmid DNA containing the gene of interest is treated in vitro by some mutagenesis procedure that alters the DNA either chemically or enzymatically. The mutagenized plasmid DNA is introduced into *E. coli* by transformation, and colonies containing plasmid molecules are selected by antibiotic resistance. Mutants can be made one at a time, or hundreds of different mutants can be created in a single mutagenesis experiment. Mutant plasmids can be isolated from single colonies and tested individually. Alternatively, plasmid DNA can be prepared from pooled colonies and the resulting library tested en masse to identify mutant plasmids.

The various approaches to mutagenesis can be grouped broadly into random and site-directed methods. Random methods put mutations anywhere in a plasmid. They are best used to identify the location and boundaries of a particular function within a cloned DNA fragment and are most readily used for this purpose when a simple genetic screen (or selection) is available. A genetic screen or selection consists of a system to test the function of the DNA of interest in cells without having to isolate each plasmid individually. Random mutagenesis is often used as a first step, when little is known about the function encoded by particular DNA fragment. Analysis of random mutants generally provides only a simple identification of the functional region but does not explain how things work on a molecular level. The value of such a strategy is that it quickly helps to narrow down the focus of attention from a large DNA fragment to a smaller region that can be studied subsequently in greater detail. As we will learn, random mutagenesis can be accomplished by several different methods, such as altering the sequences within restriction en-

donuclease sites, inserting an oligonucleotide linker randomly into a plasmid, damaging plasmid DNA in vitro with chemicals, or incorporating incorrect nucleotides during in vitro DNA synthesis.

Once an important functional domain in a gene has been identified by random mutagenesis, site-directed methods—putting mutations precisely where they are needed—are used to define the role of specific sequences. In addition, directed mutagenesis provides a powerful tool for the analysis of protein function, by allowing researchers to make specific and subtle changes in the structure of the protein. A number of strategies have been developed to construct site-directed mutants in vitro, but this type of mutagenesis is best accomplished using synthetic oligonucleotides. With an oligonucleotide the desired sequence is simply built into the wild-type framework. Nowadays, oligonucleotide-directed mutagenesis reactions are relatively straightforward, and oligonucleotides are cheap and easy to obtain. The limitation of site-directed mutagenesis is that you must already have enough information to know what you wish to change. There are two standard ways of using oligonucleotides to construct site-directed mutants: mutagenesis by gene synthesis and mutagenesis by enzymatic extension of a mutagenic oligonucleotide. By using degenerate oligonucleotides (see Chapter 7) a set of "random" mutations at a specific site can also be made.

Restriction Endonuclease Sites Provide the Simplest Access for Mutagenesis

One of the first experiments done with a cloned DNA fragment is to map the positions of restriction endonuclease cleavage sites in the DNA by using a battery of different enzymes. Although this information could be precisely obtained from the DNA sequence, mapping restriction sites can be accomplished rapidly and is often done in conjunction with sequencing. Restriction endonuclease recognition sites provide the simplest way to modify a DNA clone in vitro (Figure 11-2). Cleaving plasmid DNA with a restriction enzyme that recognizes only one site produces a linear molecule. This serves as an entry point for modifying the DNA sequence in the vicinity of the restriction site. For example, the enzyme *Eco*RI recognizes the sequence GAATTC and produces ends with 5′ overhangs. The ends can be made even (blunt) by treating

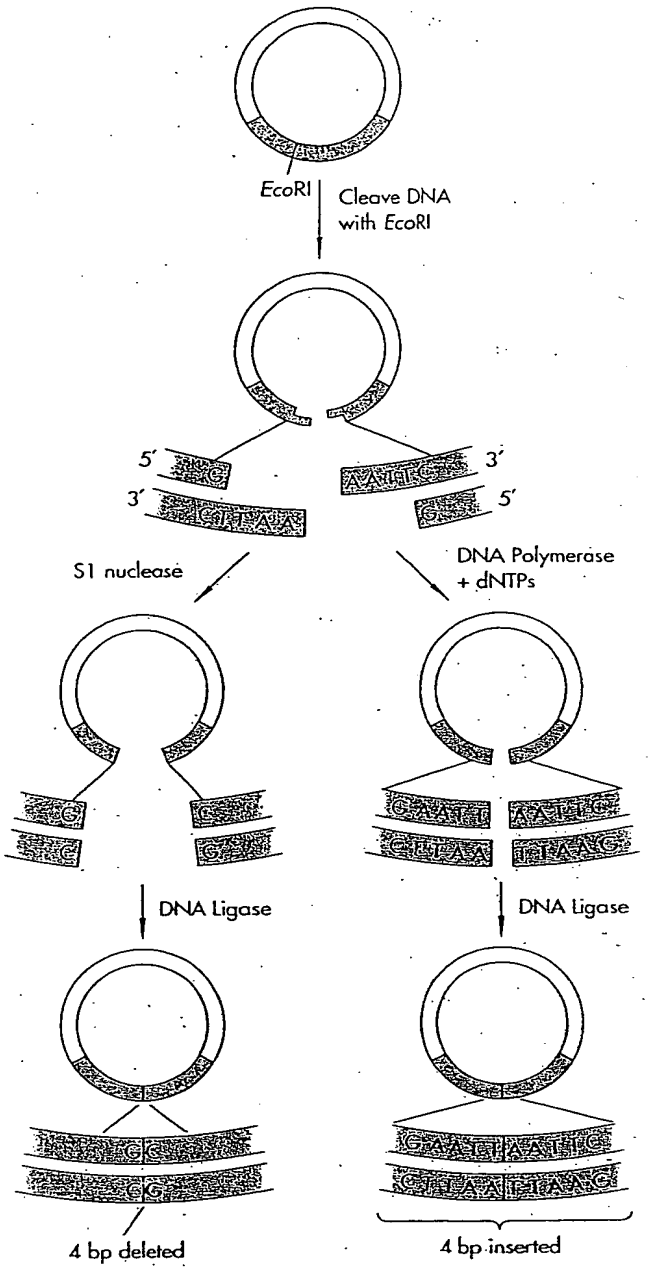
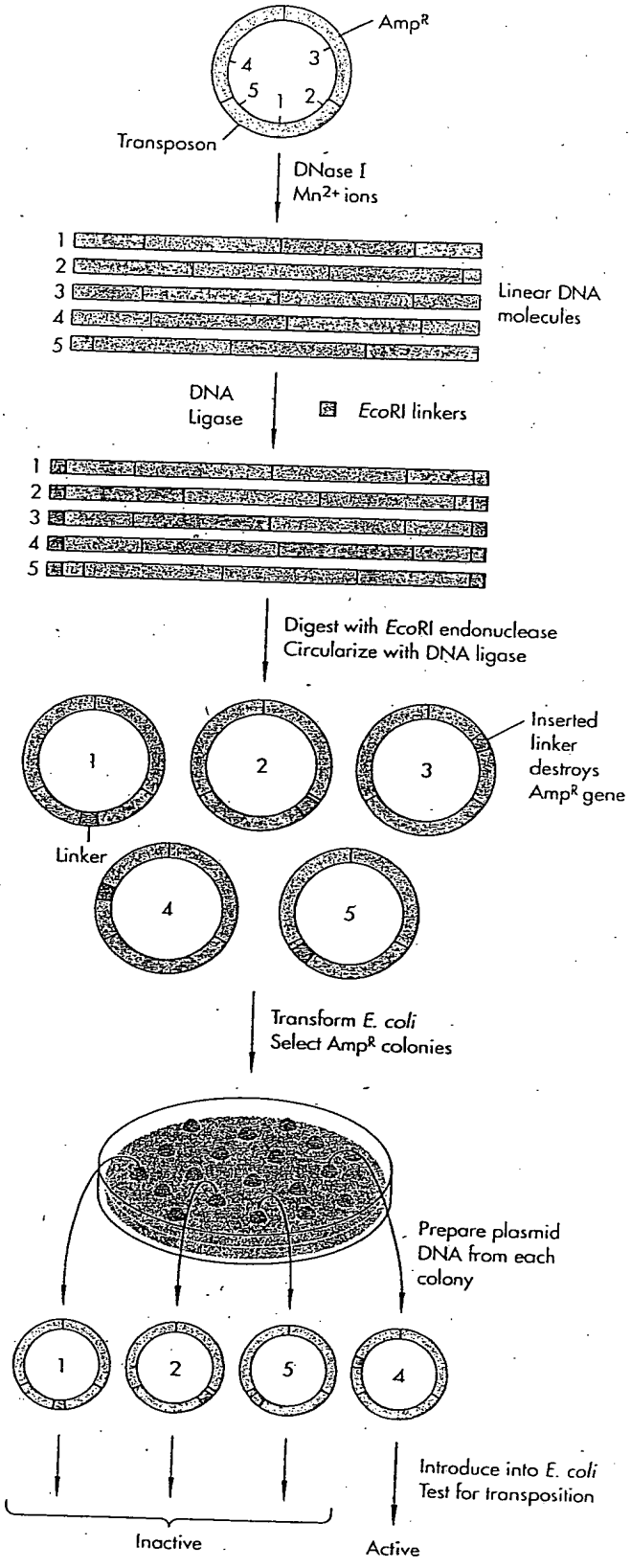


FIGURE 11-2
Creating a mutation by manipulation of a restriction site. Plasmid DNA is cleaved with *Eco*RI restriction endonuclease, which generates a linear fragment with 5′ ends that have four unpaired nucleotides (so-called sticky ends). Treatment with S1 nuclease (left) removes these nucleotides, and the linear fragment is then treated with DNA ligase. The resulting circular molecule contains a deletion of 4 bp. Alternatively, addition of DNA polymerase and deoxyribonucleotide triphosphates (dNTPs) to the plasmid cleaved by *Eco*RI extends the 3′ ends by DNA synthesis (right). After ligation, the resulting molecule contains an insertion of 4 bp. In both cases, the *Eco*RI site has been destroyed.

FIGURE 11-3
Linker insertion mutagenesis to map functional domains of a bacterial transposable element. The starting plasmid contains an intact transposon, an ampicillin-resistance gene for selection in *E. coli*, and sequences for plasmid replication. The DNA is treated with a low concentration of deoxyribonuclease I in the presence of Mn^{2+}. Under these conditions, the enzyme makes double-stranded cuts at random positions in the plasmid, generating a collection of linear DNA molecules broken at different positions. Oligonucleotide linkers encoding an *Eco*RI restriction site are added to the ends with DNA ligase, the linear molecules are treated with *Eco*RI endonuclease to create sticky ends on the linkers, and the molecules are recircularized. The circular molecules are transformed into *E. coli*, and ampicillin-resistant colonies are selected. Plasmid DNA is isolated from individual colonies, introduced into another strain of *E. coli*, and tested for activity of the transposon. The positions of the inserted linkers are mapped by restriction digestion. Linkers inserted in one region (blue) of the plasmid inactivated the transposon. No linker insertions in the ampicillin-resistance gene were recovered, because these plasmids would fail to yield a drug-resistant colony in the original selection of transformed *E. coli*.



the cleaved DNA with DNA polymerase in the presence deoxyribonucleotide triphosphates. The two blunt ends can then be linked together again (ligated) by incubating the linear plasmid molecule with DNA ligase. A few nanograms of DNA from the in vitro ligation reaction is used to transform *E. coli*, and the new modified plasmid is isolated from one of the resulting colonies. The net result of these manipulations is to insert 4 bp into the plasmid at the *Eco*RI site. Alternatively, a small deletion mutation can be made by treating the linearized DNA with S1 nuclease, which specifically digests single-stranded DNA. This creates blunt ends by removal of the four nucleotides that constitute the 5′ overhang generated by *Eco*RI at each end. Subsequent ligation of the DNA into a covalently closed circular molecule thus results in the deletion of 4 bp from the DNA. In each example, the new sequence no longer encodes the *Eco*RI recognition site. These types of manipulations, if done to a protein-coding sequence, would change the translational reading frame, resulting in production of a grossly altered protein. The major limitation of using restriction sites to make mutations is that there simply may not be sites in regions of the gene the experimenter wishes to alter.

Linker Insertion Is Used to Map a Bacterial Transposon

We have learned that it is a simple matter to cleave a plasmid with a restriction enzyme, blunt the ends by treatment with a DNA polymerase, and rejoin them by ligation. A variation on this technique is to rejoin the ends in the presence of a synthetic oligonucleotide "linker," often one that encodes a restriction site. Insertion of the linker disrupts the gene sequence; the position of the inserted linker can be easily mapped by cleavage of the plasmid with the restriction enzyme that cuts the linker.

A similar method was used to define the functional regions of a bacterial transposable element (a "jumping gene," see Chapter 10), by inserting linkers at many alternative positions throughout the element. To place linker insertions in the transposon, a plasmid carrying a clone of the transposon was treated with a nuclease that cleaved the plasmids at random positions (Figure 11-3). Cleavage conditions were adjusted so that each plasmid was cut just once on average. The linearized molecules were isolated and ligated into circles again in the presence of an 8-bp linker oligonucleotide containing an *Eco*RI restriction site, resulting in insertion of the linkers into random sites, one in each plasmid. The resulting plasmids were transformed into *E. coli* and, using a genetic screen, examined to see if the transposon could jump. Insertion of a linker into a region of the transposon critical for its function inactivates it, presumably by putting a protein-coding sequence out of frame. By mapping the positions of the inserted linkers by restriction analysis, the locations of functional regions of the transposon were deduced.

Construction of Nested Deletions Maps the Boundaries of a Transcriptional Control Region

Transcription of the gene encoding the 5S ribosomal RNA molecule is carried out by RNA polymerase III (pol III, see Chapter 8). To identify the sequences within the 5S gene required for transcription by pol III, a series of deletion mutations was made and tested for their ability to support accurate transcription. Two sets of deletions were made. One was made by cutting a plasmid carrying a cloned 5S gene at a restriction site on the 5′ side of the gene. The linearized plasmid was treated with a combination of nucleases that digested away DNA from the ends of the molecule (Figure 11-4). The amount of DNA removed was controlled by varying the time, temperature, or enzyme concentration in the reaction. A second set of deletions was generated from plasmid DNA cleaved at a site on the 3′ side of the gene. The result was two sets of plasmids with progressively larger deletions toward the gene from both directions. Testing these genes revealed that only deletions entering a 35-bp region within the transcribed region of the 5S gene abolished transcription by pol III. Therefore, this deletion analysis mapped the transcriptional regulatory element to this 35-bp stretch, which has subsequently been analyzed in much greater detail by site-directed mutagenesis.

Several different types of enzymes can be used to produce deletions. Generally, these enzymes delete DNA from both ends of a linearized plasmid molecule. Often, however, one end of the molecule contains sequences that need to be retained in the plasmid because, for example, they are required for plasmid replication. In the 5S gene deletion experiment, this limitation was accommodated by isolating the deleted gene fragments and recloning them into a new vector. Alternatively, a strategy can be used that limits deletion to one end of a linearized plasmid molecule (Figure 11-5). This method is widely used to generate nested deletions for DNA sequencing (see Chapter 7).

Linker-Scanning Mutagenesis Permits Systematic Analysis of Promoters

Deletion mutagenesis of the 5S gene mapped the boundaries of the transcriptional control region in the gene. But not all the nucleotides within the boundaries of that 35-bp region are necessarily critical for function. Therefore, methods were needed to change individual nucleotides in a target without generating gross deletions or other rearrangements. This was accomplished for a viral promoter using an elegant ad-

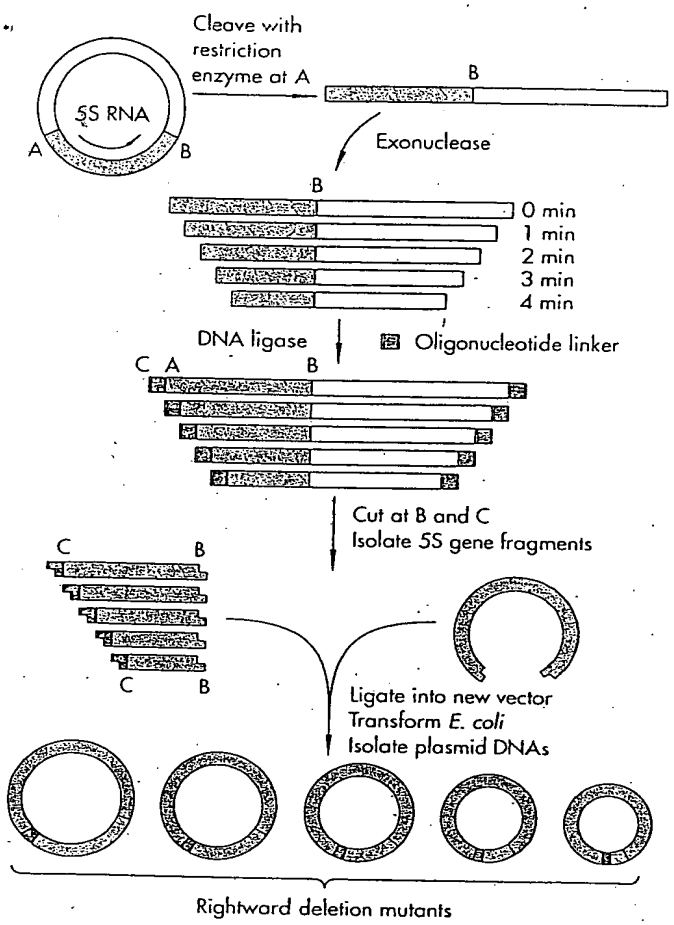
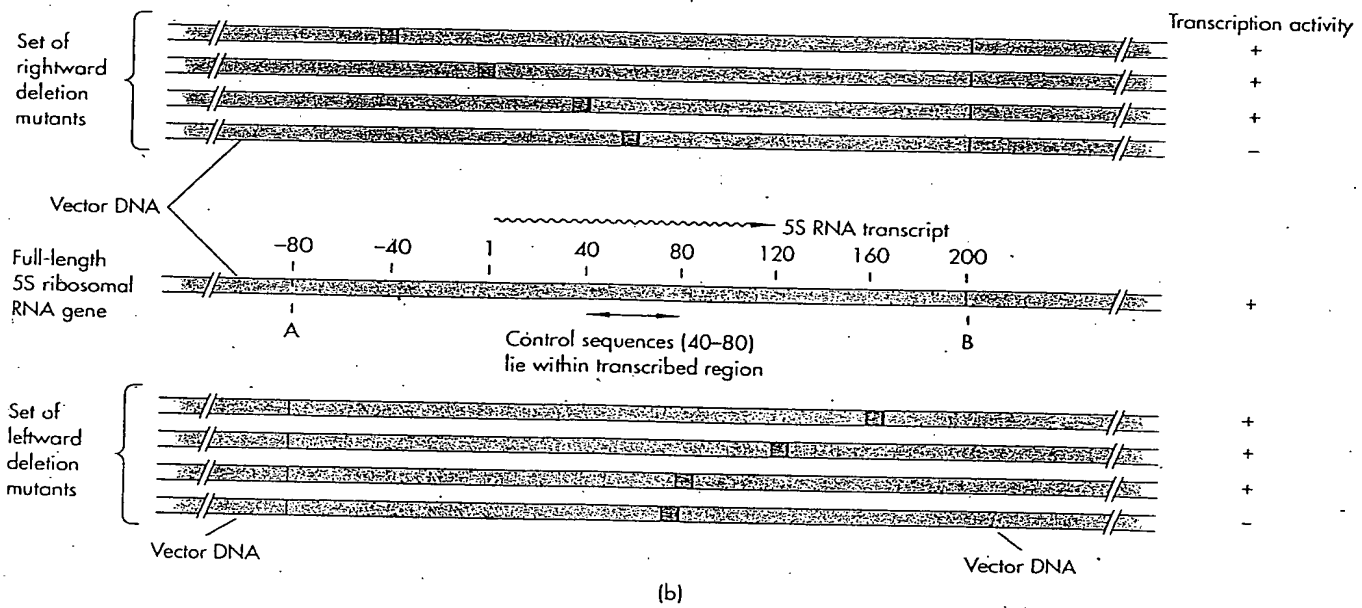


FIGURE 11-4
Construction of a nested set of deletion mutants to map the transcription control region of a 5S ribosomal RNA gene. (a) A plasmid clone was linearized with a restriction enzyme at a position (A) on the 5′ side of the gene. The linear fragments were treated with an exonuclease, which digests DNA from both ends of the molecule. Portions of the reaction were removed at different times to recover populations of molecules with progressively larger deletions. Linkers were added to the ends, and the molecules were cleaved with restriction enzymes specific for sites B and C to separate the 5S gene fragments from the remnants of the vector. The fragments were recloned into a new vector, generating the set of rightward deletion mutants. To create the leftward deletion mutants, this process was repeated after cleaving the plasmid at restriction site B. (b) Individual plasmids were isolated after transformation, their deletion endpoints determined by DNA sequencing, and their ability to support transcription by RNA polymerase III tested with an in vitro assay. As can be seen by comparing transcription activity with the extent of deletion, transcription is inhibited when the rightward (5′) deletions enter the +40 region and when the leftward (3′) deletions pass the +80 point. This suggests that the transcription control region lies between +40 and +80.



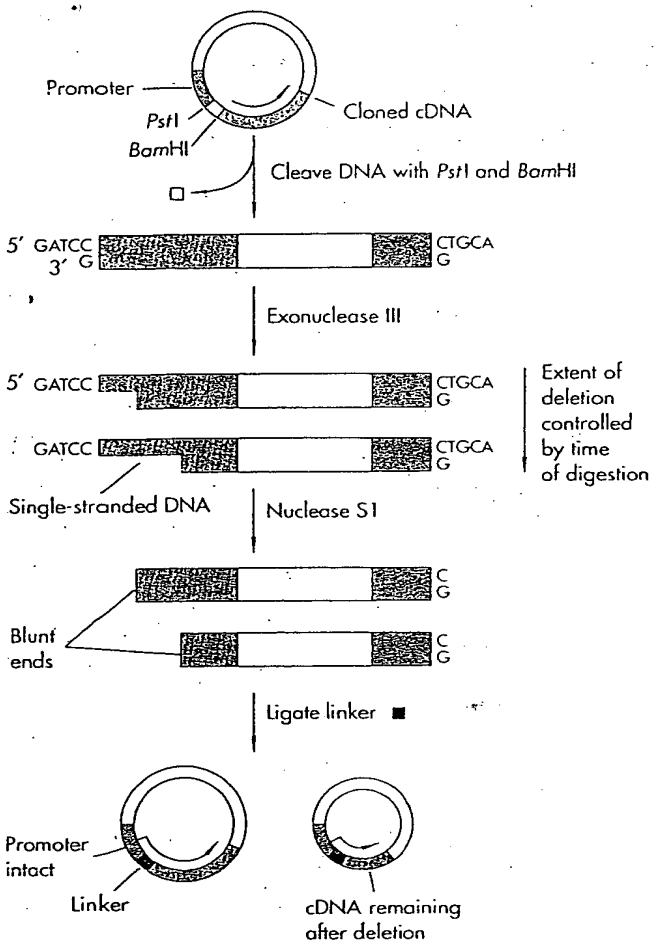


FIGURE 11-5
Construction of unidirectional deletions using exonuclease III. Exonuclease III attacks preferentially the 3′ end of a linear DNA molecule with 5′ protruding nucleotides. Therefore, by cleaving a plasmid molecule at adjacent sites with *Bam*HI, which leaves a 5′ overhang, and *Pst*I, which leaves a 3′ overhang, only the end generated by *Bam*HI is attacked by exonuclease III. After exonuclease III treatment, the remaining single-stranded tail (along with the overhang at the other end) is removed with S1 nuclease, which digests only single-stranded DNA. An oligonucleotide linker is attached, and the fragments are ligated to form closed circular molecules. In the experiment shown here, deletions are being used to map the functional domains of a cloned gene inserted in an expression vector. This strategy allows deletions to be made only in the cloned gene, without damaging the promoter sequence.

aptation of deletion mutagenesis called *linker scanning*. Using the methods outlined in Figure 11-4, two sets of plasmids were constructed that contained deletions within the promoter. One set of deletions started from a site beyond the 5′ end and proceeded toward the gene, leaving the 3′ end intact; the other set started at a point within the gene and proceeded in the opposite direction, leaving the 5′ end intact. Each deletion terminated with a 10-bp *Bam*HI linker. The extent of the deletion in the DNA was determined for each plasmid by DNA sequencing. Pairs of plasmids from the two deletion sets with endpoints 10 bp apart were recombined at their *Bam*HI sites (Figure 11-6). The effect was to preserve the length and organization of the promoter—thought to be important for promoter function—but to replace various 10-bp segments of wild-type promoter sequence with the sequence in the linker. Thus, this experiment created a library of promoter mutants of similar structure but with nucleotide substitutions clustered within 10-bp windows located at various sites in the promoter. This collection of mutants spanned the length of the promoter. The results of this analysis were discussed in Chapter 9. At the time, this experiment represented the most thorough analysis of a promoter in a mammalian gene.

Random Nucleotide Substitutions Are Obtained by Chemical Modification of DNA or by Enzymatic Misincorporation

While linker scanning allows the creation of nucleotide substitutions, each mutant generally contains several substitutions, and the positions of the mutations depend on the availability of appropriately placed deletions. Therefore, several strategies have been developed for placing *single* nucleotide substitutions at random positions in a DNA molecule. The simplest methods employ chemicals that modify or damage DNA. Generally, plasmid DNA or DNA fragments are treated with chemicals, transformed into *E. coli*, and propagated as a library of mutant plasmids. Chemicals most commonly used for in vitro mutagenesis include sodium bisulfite, which deaminates cytosine residues to uracil, and reagents that damage or remove

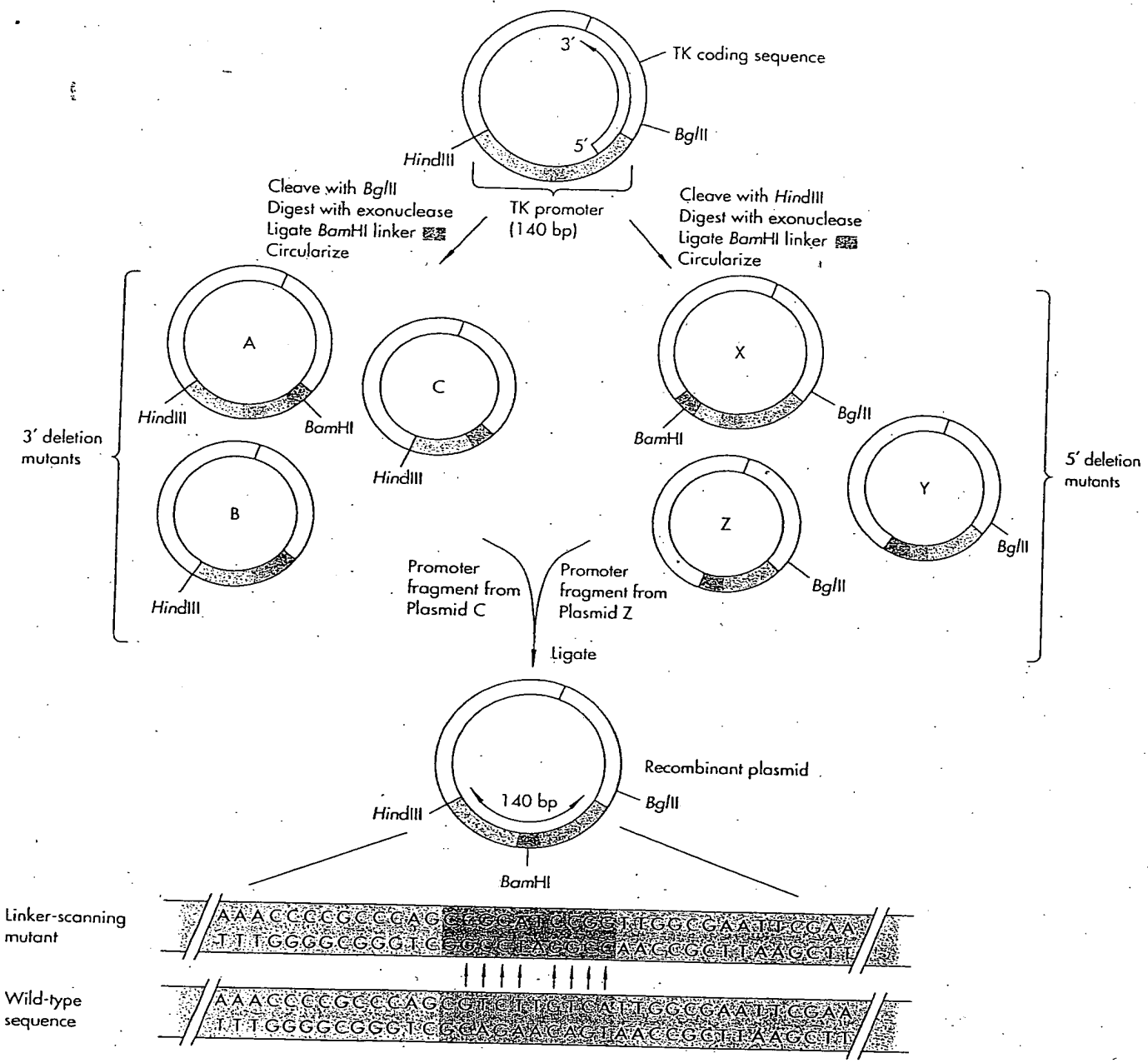


FIGURE 11-6
Linker-scanning mutagenesis of the viral promoter for the thymidine kinase (TK) gene. Two sets of deletion mutants were made beginning from restriction sites on the 5′ and 3′ sides of the promoter by the method described in Figure 11-4. (The promoter is divided into three colors to make the extent of deletion more obvious.) Approximately one hundred plasmids were sequenced to determine their deletion endpoints. Pairs of deletion fragments, where the 5′ deletion of one fragment ended precisely 10 bp downstream from the endpoint of the 3′ deletion of the other fragment, were identified. The *Hin*dIII–*Bam*HI fragment of the 3′ deletion mutant and the *Bam*HI–*Bgl*II fragment of the 5′ deletion mutant were joined via their *Bam*HI sticky ends and cloned into a new plasmid. This strategy yields molecules like the one shown at the bottom: they are wild-type in sequence except for the substitution of the 10-bp *Bam*HI linker in place of the sequence between the two deletion endpoints. In the example shown, this results in a cluster of eight nucleotide substitutions (arrows).

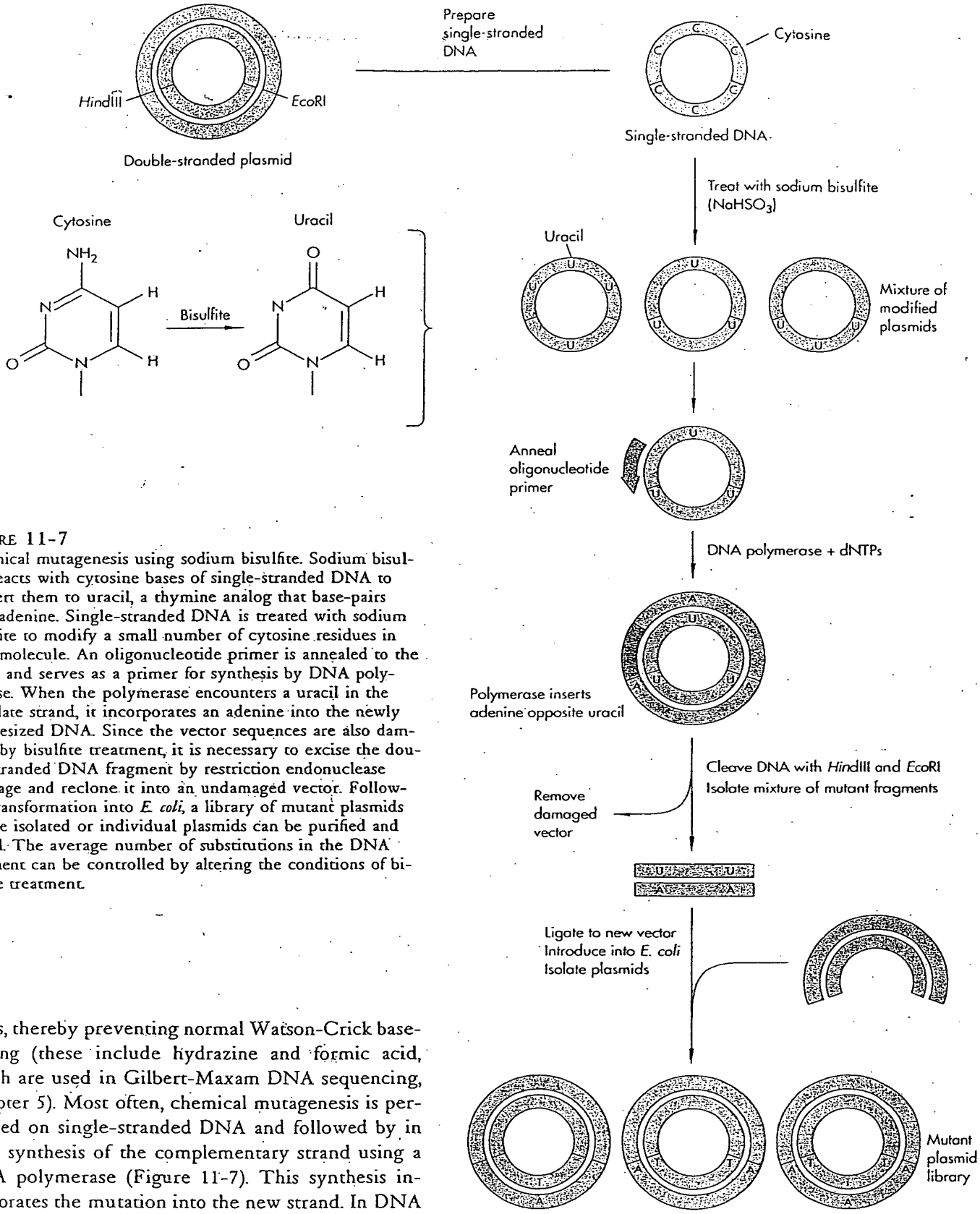


FIGURE 11-7
Chemical mutagenesis using sodium bisulfite. Sodium bisulfite reacts with cytosine bases of single-stranded DNA to convert them to uracil, a thymine analog that base-pairs with adenine. Single-stranded DNA is treated with sodium bisulfite to modify a small number of cytosine residues in each molecule. An oligonucleotide primer is annealed to the DNA and serves as a primer for synthesis by DNA polymerase. When the polymerase encounters a uracil in the template strand, it incorporates an adenine into the newly synthesized DNA. Since the vector sequences are also damaged by bisulfite treatment, it is necessary to excise the double-stranded DNA fragment by restriction endonuclease cleavage and reclone it into an undamaged vector. Following transformation into *E. coli*, a library of mutant plasmids can be isolated or individual plasmids can be purified and tested. The average number of substitutions in the DNA fragment can be controlled by altering the conditions of bisulfite treatment.

bases, thereby preventing normal Watson-Crick base-pairing (these include hydrazine and formic acid, which are used in Gilbert-Maxam DNA sequencing, Chapter 5). Most often, chemical mutagenesis is performed on single-stranded DNA and followed by in vitro synthesis of the complementary strand using a DNA polymerase (Figure 11-7). This synthesis incorporates the mutation into the new strand. In DNA treated with bisulfite, an adenine nucleotide is incorporated opposite the uracil; after transformation into

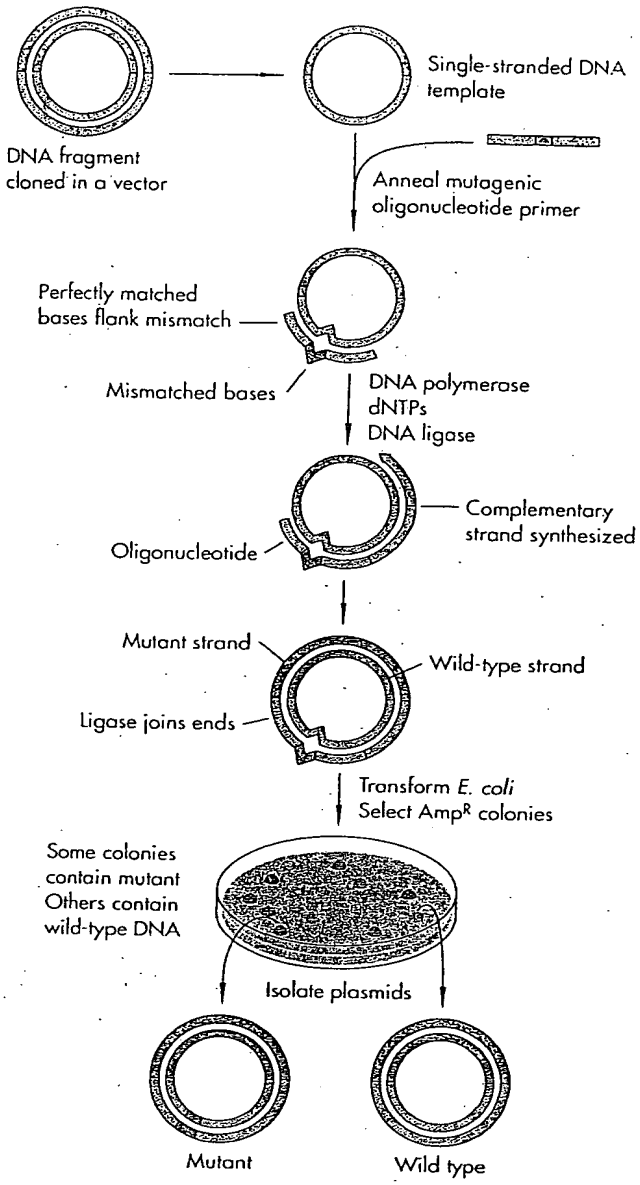


FIGURE 11-8
Oligonucleotide-directed mutagenesis by enzymatic primer extension. A "mutagenic" oligonucleotide encoding the desired mutation embedded in wild-type flanking sequence is annealed to a single-stranded DNA template. The sequence of the oligonucleotide is complementary to the template except for the nucleotides that define the mutation. Generally, the mutagenic oligomer is designed so that the mismatched nucleotides are positioned in the middle and there are at least 8 to 12 nucleotides on either side that base-pair with the template DNA. The mutagenic oligonucleotide serves as a primer for DNA synthesis by DNA polymerase. Once the entire template has been copied, the ends of the newly synthesized strand are covalently linked by DNA ligase. The heteroduplex DNA is transformed into *E. coli*. Theoretically, both strands can replicate, segregating into separate mutant and wild-type plasmids. In practice, however, most colonies contain only one or the other, because enzymes in the cell recognize and repair mismatched nucleotides in the heteroduplex before replication. Plasmid DNA is isolated from the resulting colonies and is screened to identify mutants.

E. coli, the wild-type C·G base pair becomes a T·A pair. In DNA treated with reagents that eliminate bases, any nucleotide can be incorporated opposite the "abasic" site, which still retains its deoxyribose backbone although it has lost its base. The major limitation of chemical mutagenesis is the specificity of the individual reagents: bisulfite mutagenesis, for example, changes only cytosines.

All possible nucleotide substitutions can be generated using enzymatic misincorporation. Here the strategy is to perform in vitro DNA synthesis under nonideal conditions—suboptimal ionic conditions, unbalanced concentrations of nucleotide precursors—that encourage DNA polymerase occasionally to incorporate the wrong nucleotide during synthesis. For example, synthesis is carried out in the presence of high concentrations of three of the precursors and a very low concentration of the fourth. At positions that normally call for the fourth (scarce) nucleotide, one of the others is sometimes incorporated instead. These methods also exploit DNA polymerases that lack a proofreading activity—a 3′ to 5′ exonuclease mechanism that checks each base pair after incorporation and removes nucleotides that are mismatched. *Thermus aquaticus (Taq)* DNA polymerase, used in the polymerase chain reaction (Chapter 6), lacks such an activity. Though this is a problem when accuracy of synthesis is required, the PCR is a very simple and efficient way to introduce random nucleotide substitutions into a DNA fragment.

A general problem with random mutagenesis approaches is that they often produce mutants with more than one substitution. Multiple substitutions in a single mutant complicate the interpretation of an experiment, because it isn't clear which substitution (or which combination of substitutions) is responsible for

observed changes in the properties of the mutant. Extraordinary methods have been used to circumvent this problem—essentially, significantly reducing the extent of mutagenesis and using enrichment protocols to find rare mutants—but almost all these procedures have been supplanted by new methods that use synthetic oligonucleotides.

Synthetic Oligonucleotides Facilitate Mutagenesis

Most of the methods for mutagenesis we have discussed so far have some significant shortcoming—they rely on fortuitous access to a sequence via a restriction site, forced entry through deletion strategies, or tedious screens to find randomly generated mutations in the region of interest. To be most powerful, mutagenesis must allow the experimenter to place *any* modification at *any* position desired in cloned DNA. This has become not only possible, but simple and cheap, with the advent of synthetic DNA oligonucleotides. Oligonucleotides provide the means to design a particular mutation and then to place it precisely where you want it.

The simplest method for doing oligonucleotide-directed mutagenesis is by enzymatic primer extension (Figure 11-8). In this method, an oligonucleotide is designed that carries the mutation flanked by 10 to 15 nucleotides of wild-type sequence. This "mutagenic" oligonucleotide is hybridized to its complementary sequence in single-stranded wild-type DNA prepared from a phage or phagemid clone, forming a heteroduplex with mismatched nucleotides at the site of the mutation. Although the oligonucleotide is not perfectly complementary, it will anneal if the hybridization conditions are not very stringent. The oligonucleotide serves as a primer for in vitro enzymatic DNA synthesis by a DNA polymerase that converts the single-stranded DNA into double-stranded form, using the wild-type strand as template. In this way, all regions of the plasmid except the region containing the mutagenic oligonucleotide will be wild-type in sequence. Once the primer has been extended completely around the template, the ends of the newly synthesized strand are ligated, forming a double-stranded circular DNA molecule. This heteroduplex DNA—one strand has the wild-type sequence and the other strand has the mutant sequence—is transformed into *E. coli*, where either strand can be replicated. By the time a colony grows up, however, it usually contains only one type of plasmid, wild-type or mutant. The types of mutations that can be made by this approach range from single nucleotide substitutions to deletions or insertions, limited only by the size of the oligonucleotide needed.

Mutant Clones Can Be Identified by Hybridization and DNA Sequencing

Theoretically, half the daughter molecules of a mutagenesis reaction will be wild-type and half mutant. In practice, however, the precentage of mutant plasmids is often much lower. This is due to a variety of technical factors, but the consequence is that methods for identifying or enriching mutant clones are vital. Mutant molecules can be distinguished from wild-type if there is gain or loss of a restriction site. Alternatively, the oligonucleotide that was originally used to make the mutation can be used as a hybridization probe to distinguish mutant from wild-type molecules (Figure 11-9). The mutagenic oligonucleotide is radioactively labeled with ^{32}P-ATP and hybridized to DNA from bacterial colonies on nitrocellulose filters, as described in Chapter 7. If the temperature of the hybridization is raised in 5 or 10°C increments, a point can usually be reached at which the labeled oligonucleotide will hybridize only to the mutant molecules (to which it is perfectly complementary) and not to the wild-type molecules, because the hybrid is destabilized by the mismatched nucleotides. Plasmid DNA is isolated from an *E. coli* colony that strongly hybridizes to the probe. Verification that the desired mutation was made is accomplished by sequencing the DNA of this putative mutant clone. This technique can identify one mutant clone among several hundred wild-type clones.

Several clever methods enrich for mutant clones so that the tedious task of screening by hybridization is not necessary. In one of these techniques, the template DNA is biologically marked so that it is destroyed after transformation into *E. coli* and the mutant strand

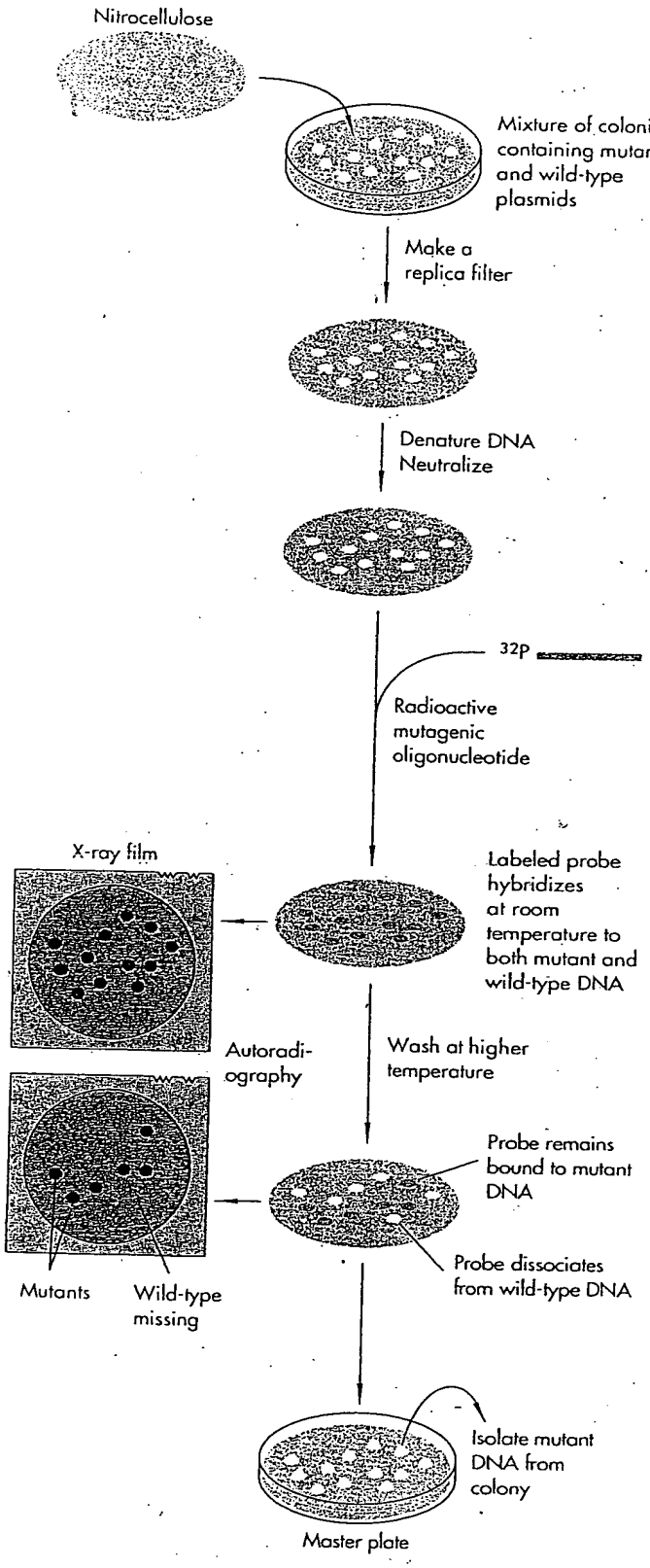


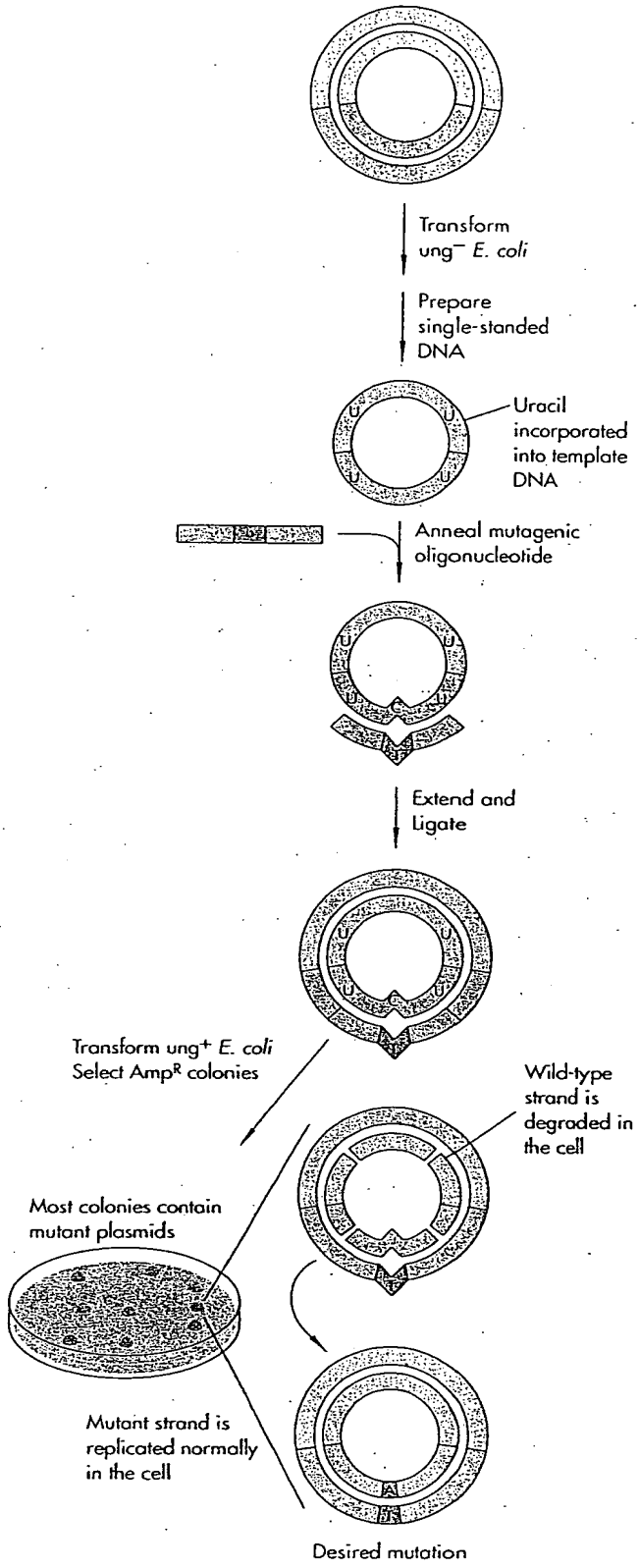
FIGURE 11-9
Searching for mutant plasmids using the mutagenic oligonucleotide as a probe. Colonies (or plaques) resulting from transformation by mutagenized plasmids (see Figure 11-8) are prepared for colony hybridization on nitrocellulose filters using methods described in Chapter 7. The mutagenic oligonucleotide is radioactively labeled by phosphorylating its 5′ end using ^{32}P-ATP and polynucleotide kinase. The labeled oligonucleotide is hybridized to the plasmid DNA on the nitrocellulose filters. At low temperature, the oligonucleotide will hybridize to both mutant and wild-type DNAs. As the temperature is increased, the mismatched oligonucleotide hybridized to the wild-type plasmid DNA begins to dissociate from the wild-type clones. Eventually a temperature is reached at which the mismatched oligomers completely dissociate from the wild-type clones but remain hybridized to the mutants. Since the oligonucleotide is radioactively labeled, the nitrocellulose filter is exposed to x-ray film and mutant clones are identified by the presence of a strong signal on the autoradiograph. Mutant plasmid DNA is then isolated from the corresponding colony on the master plate, using the replica filter as a guide.

is preferentially replicated (Figure 11-10). In a second method, the template strand is enzymatically destroyed before transformation. Both methods can yield mutants at a frequency of greater than 50 percent, so that plasmid DNA is simply isolated from three or four randomly picked colonies and analyzed by DNA sequencing with the expectation that a mutant will be found among the DNA selected.

Oligonucleotide Cassettes Provide a Simple Method for Introducing Directed Mutations

We learned earlier that restriction enzyme sites provide access to a cloned DNA for mutagenesis. If two restriction sites are close together, the intervening fragment can be removed and replaced with a synthetic double-stranded fragment (a *cassette*) made from two complementary single-stranded oligonucleotides carrying any desired sequence. Often, however, convenient restriction sites are not available; fortunately, it is a simple matter to create them using the oligonucleotide-directed mutagenesis procedures described in the previous sections. Once the sites are in place, any

FIGURE 11-10
Enrichment for oligonucleotide-directed mutants by using a uracil-containing template. Single-stranded template DNA is prepared in a strain of *E. coli* that lacks the enzyme uracil deglycosidase (ung$^-$), so that it contains several uracil residues in place of thymines. (Although uracil is not usually incorporated into DNA, it is not actually mutagenic and it does form a base-pair with adenine.) The mutagenic oligonucleotide is annealed and primes the synthesis of a strand that extends around the template in a reaction using the four standard dNTPs (as in Figure 11-8). Following ligation, the heteroduplex DNA molecules are introduced into an ung$^+$ strain of *E. coli*. Once in the cell, the wild-type (template) strand is attacked by uracil deglycosidase, which causes breaks in the DNA strand, and the DNA strand is degraded before it can be replicated. Since the strand containing the mutagenic oligonucleotide does not contain uracil, it is not attacked and is replicated normally. When this procedure is used, 50 percent or more of colonies contain mutant plasmids.



number of new mutants can be made by inserting synthetic fragments into the plasmid (Figure 11-11), just as different cassettes can be inserted into a tape player.

This method of cassette mutagenesis was the basis for an elegant experiment that verified a structural model for DNA recognition by phage repressors. The repressors of the λ-like phages 434 and P22 contain a helix-turn-helix structure (see Chapter 9) that recognizes the operator DNA in the phage genome. It was hypothesized that amino acid side chains on one face of an α helix in the repressor protein make sequence-specific contacts with operator DNA. To test this hypothesis, a *helix swap* was performed (Figure 11-12). Oligonucleotides were synthesized that encoded the amino acids of the helix in the 434 repressor, with the five positions thought to contact DNA changed to those found in the P22 repressor. This synthetic fragment was swapped for the natural fragment in the 434 gene. The resulting hybrid protein gained the recognition specificity of the P22 repressor, demonstrating that this helix indeed contacts the DNA.

Cassette mutagenesis with degenerate oligonucleotides can be used to create a large collection of random mutations in a single experiment. This method was

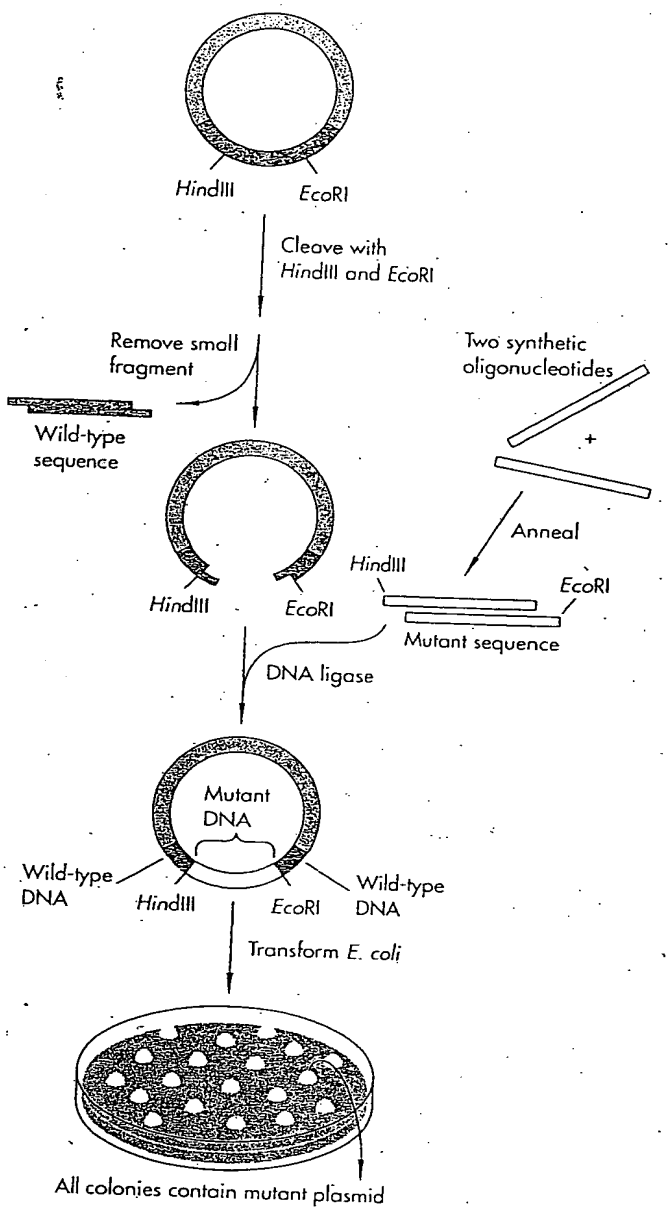


FIGURE 11-11
Mutagenesis by cassette replacement. Plasmid DNA is cleaved with restriction enzymes *Eco*RI and *Hin*dIII, which cut at sites that flank the sequence to be mutated. The small cleaved DNA fragment containing a portion of the wild-type sequence is removed, and a DNA fragment (cassette) containing the desired mutation is ligated into the plasmid. This mutant DNA fragment is composed of two complementary synthetic oligonucleotides that have *Eco*RI and *Hin*dIII sticky ends when annealed. Because there is no heteroduplex intermediate—the mutant cassette is simply swapped for the wild-type fragment—the recombinant plasmids are all mutants. A mutant cassette can be composed of degenerate oligonucleotides (see Chapter 7), resulting in a library of mutant plasmids containing different sequences.

used to study the structure of the glucocorticoid response element (GRE), an enhancer sequence that activates a family of genes in response to certain steroid hormones. The element had been mapped by deletion mutagenesis to a 30-bp region in a glucocorticoid-regulated gene. To define the sequence required for GRE function precisely, single point mutations throughout the 30-bp region were generated and tested in cells for inducibility by glucocorticoid hormone. Two complementary oligonucleotides were synthesized that carried the 30-bp GRE, but synthesis was performed under conditions in which incorrect nucleotides were incorporated at a low frequency (Figure 11-13). These "doped" oligonucleotides (that is, oligonucleotides produced by doping; see Figure 11-13) were annealed and inserted as a cassette into a promoter that lacked a GRE. Using this method, most single-nucleotide substitutions at the 30 positions were obtained. Such a collection of mutants would have been unthinkable before oligonucleotides revolutionized in vitro mutagenesis.

Gene Synthesis Facilitates Production of Normal and Mutant Proteins

The oligonucleotide-directed mutagenesis methods we have described use a single oligonucleotide or a pair of complementary oligonucleotides to insert mutant sequences into an otherwise natural DNA fragment. With the increasing availability of longer oligonucleotides, it is now feasible to assemble an entire gene from synthetic units. This is done by synthesizing a set of oligonucleotides, typically 40 to 80 nucleotides in length, that can be annealed and ligated in vitro to assemble an entire double-stranded DNA

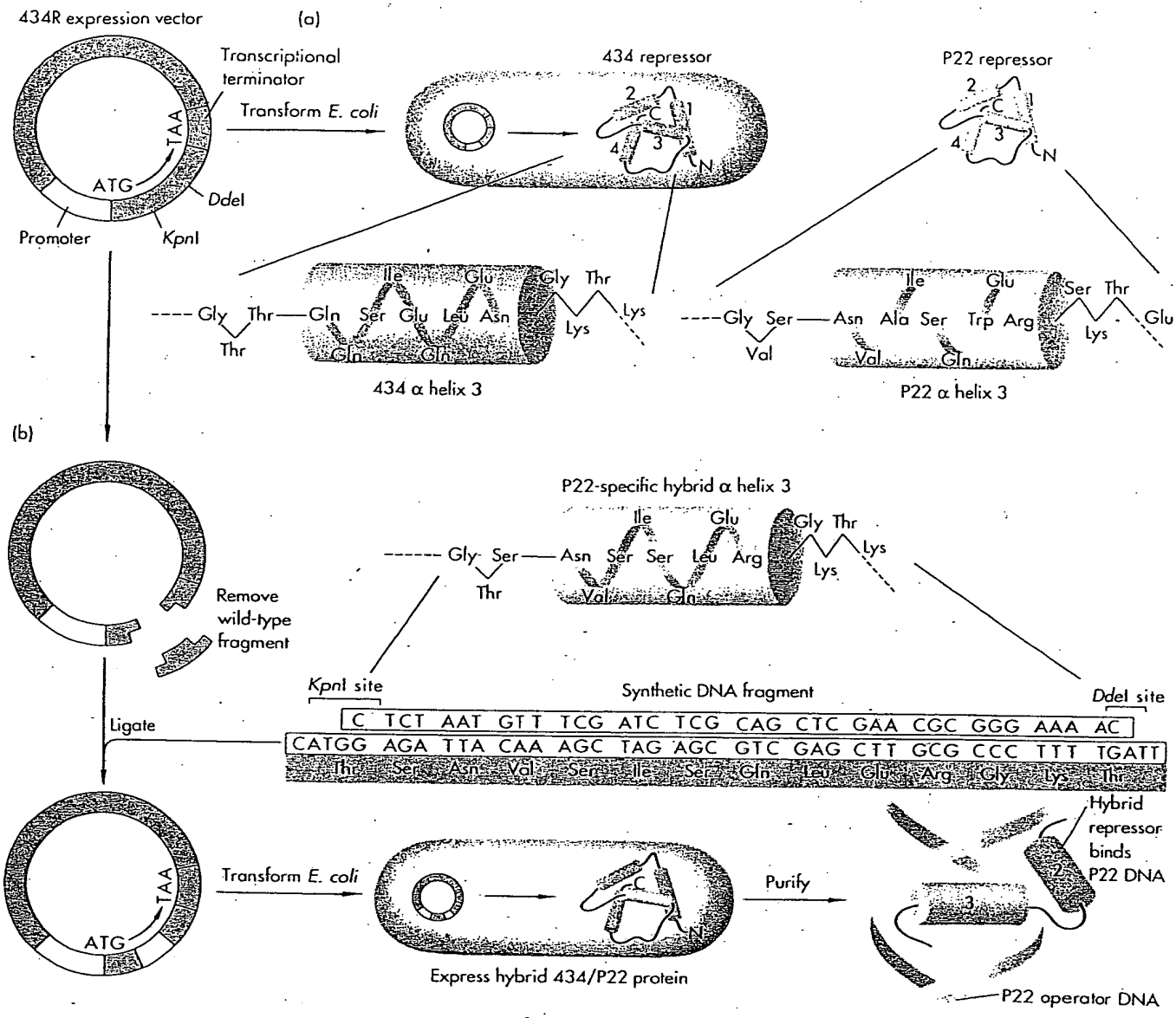


FIGURE 11-12
The helix swap experiment. Amino acids in the phage 434 repressor protein believed responsible for recognition of the 434 operator were changed by cassette mutagenesis (Figure 11-11) of 434 DNA to the amino acids believed to perform the same function in an analogous region of phage P22 repressor protein. (a) Expression in *E. coli* of the 434 repressor protein (left), with an enlargement of the site believed to bind the 434 operator; (right) the corresponding section of the P22 repressor protein. (b) A cassette was synthesized resembling the 434 domain, but with P22-type substitutions at positions thought to be essential for recognizing P22 operator DNA. This was ligated into the digested 434 plasmid, and the recombinant vector was introduced into *E. coli* to produce the hybrid protein, which then recognized P22 operator DNA but not the 434 operator.

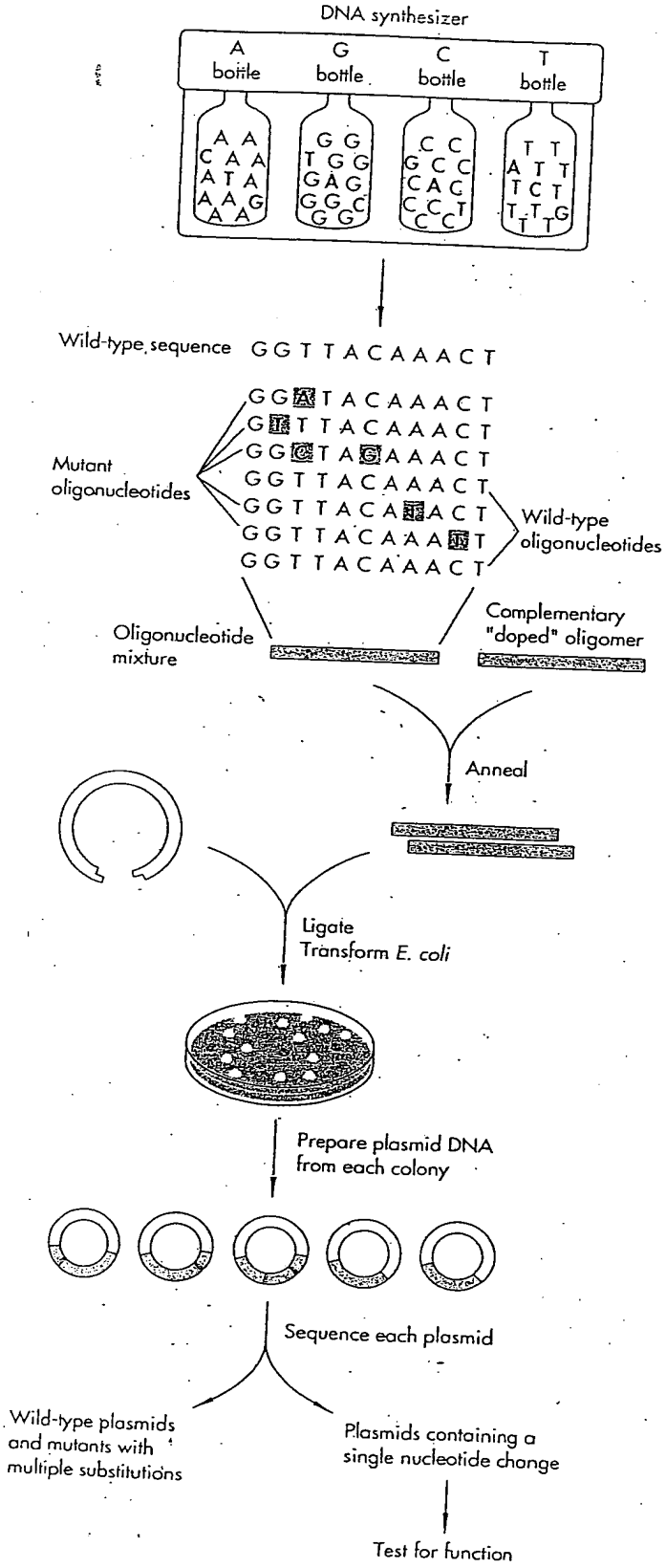
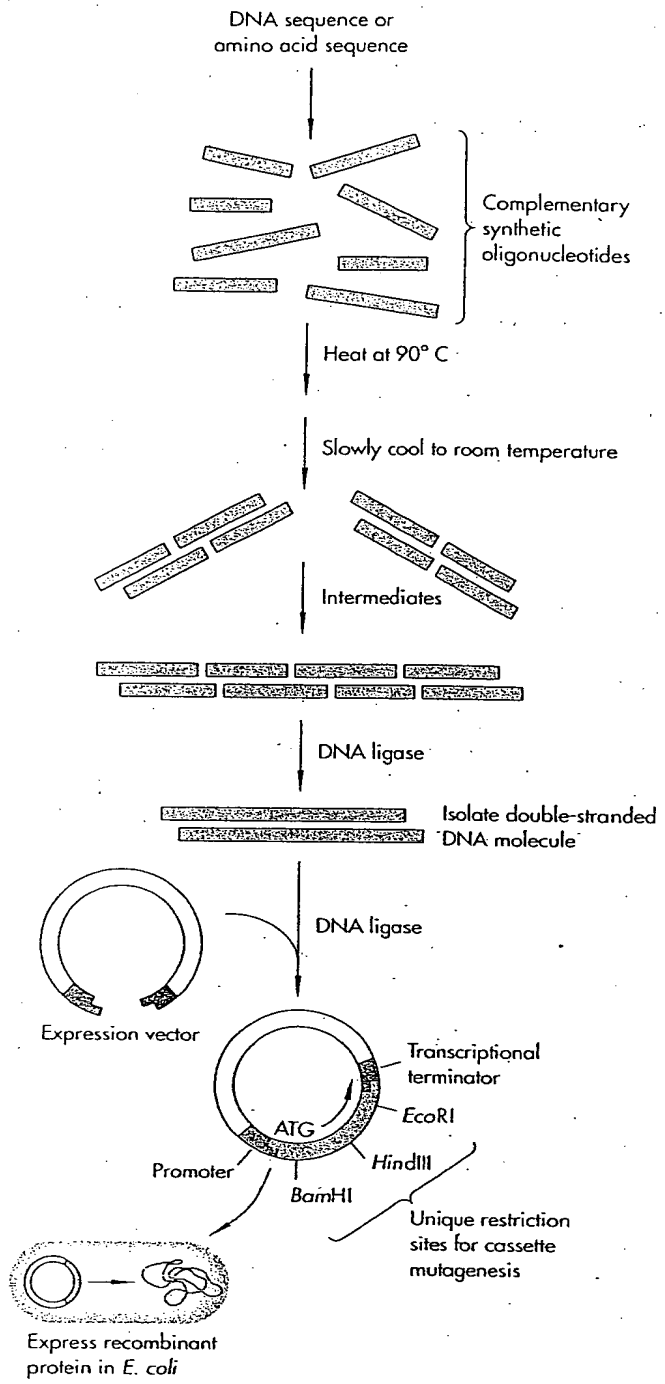


FIGURE 11-13
Cassette mutagenesis using doped oligonucleotides to generate numerous mutants in a single experiment. An oligonucleotide cassette encoding the glucocorticoid response element (GRE) was synthesized by a DNA synthesis machine. Synthesis was done under conditions in which each bottle containing a particular nucleotide precursor was "contaminated" (doped) with small amounts of the other three precursors. In the example above, the DNA synthesizer was instructed to make an oligonucleotide with the sequence GGTTACAAACT. Thus, when a nucleotide precursor is called for—a C for example—the machine adds an aliquot of the solution from the C bottle, and a C base is coupled to the end of most of the oligonucleotide chains. However, because the C bottle contains a small amount of A, G and T, an incorrect base is sometimes added instead. Since the concentration of C is roughly 30 times that of A, G and T, an incorrect base will be added to about 1 out of 30 molecules. This results in a doped collection of oligonucleotides, which actually consists of many different sequences, some wild-type and some with substitutions. The level of contamination was adjusted to favor synthesis of oligonucleotides with only one substitution, but because substitutions occur randomly, some molecules in the collection had none and others had two or more. Cassettes were formed by annealing complementary doped oligonucleotides and ligated into a vector. Plasmid DNA was isolated from 546 individual *E. coli* transformants and analyzed by sequencing. Of these, 224 were wild-type, 218 contained one substitution (for the 30 bases, of interest, 74 of the 90 possible single substitutions were recovered), and the rest contained two or more.

molecule (Figure 11-14). In gene synthesis, the experimenter has total control over the sequence of the gene. It can be wild-type or mutant in any way required. Because most amino acids are encoded by multiple triplet codons, genes encoding wild-type proteins can be constructed using different codons. Codons can be chosen to place unique restriction sites throughout the sequence so that mutant cassettes can be easily swapped in. This was done with the bacterial rhodopsin gene. Replacing a fragment of the synthetic gene with a new synthetic fragment identified the amino acid that is linked to the photon-absorbing chromophore that initiates photosynthesis. Other fragments can be exchanged as cassettes to study other important structural features of the protein.

Codons can also be changed by gene synthesis to allow production of proteins at high levels in other organisms. Studies of the biochemistry of the Fos protein, encoded by a cellular protooncogene in animal

FIGURE 11-14
Gene synthesis by ligation of complementary oligonucleotides. To synthesize a gene that encodes a protein of interest, a set of overlapping complementary oligonucleotides are designed that can be combined to form a double-stranded DNA molecule that encodes the entire protein. The oligonucleotides are mixed together, heated at 90°C for a few minutes to denature the strands, and then cooled slowly to room temperature. During this period the oligonucleotides anneal through complementary base pairs. The oligonucleotides are designed so that each one anneals to two adjacent oligonucleotides from the opposite strand, bridging them. Generally, oligonucleotides ranging in length from 40 to 80 nucleotides are used in gene synthesis. The annealed oligonucleotides are covalently linked by DNA ligase, producing two contiguous DNA strands. This synthetic gene is usually purified from a gel before ligation into a vector. The resultant recombinant plasmid is obtained following transformation into *E. coli* and is sequenced to check that the correct sequence was synthesized. The sequence of the synthetic gene can be designed to place restriction sites at convenient locations for cassette mutagenesis.



cells (Chapter 18), have been severely hampered by the inability to produce the protein in *E. coli*. This problem was finally solved by synthesizing a portion of the *fos* gene entirely from oligonucleotides, changing natural *fos* codons to the codons used most efficiently in *E. coli*. Insertion of this synthetic gene into an *E. coli* expression vector allowed for the first time the production of large quantities of active Fos protein. The gene was also designed with several unique restriction sites so that efficient cassette mutagenesis can now be coupled to the biochemical assays for Fos function.

The PCR Can Be Used to Construct Genes Encoding Chimeric Proteins

The ease with which mutations can be made in a protein coding sequence has revolutionized the study of protein function. A functional domain can be identified by making a series of mutant proteins, then testing which substitutions cause a change in function. However, it is not often easy to decide where to make a mutation. In the example of the helix swap experiment (Figure 11-12), the domain that bound DNA had been previously identified. And the design of the experiment was guided by having a model for the three-dimensional structure of the repressor protein.

However, for most proteins, little structural information is available. Identifying a functional domain—for example, a region of the protein that may interact with another protein—is difficult to do by inspecting the primary amino acid sequence. A simple strategy

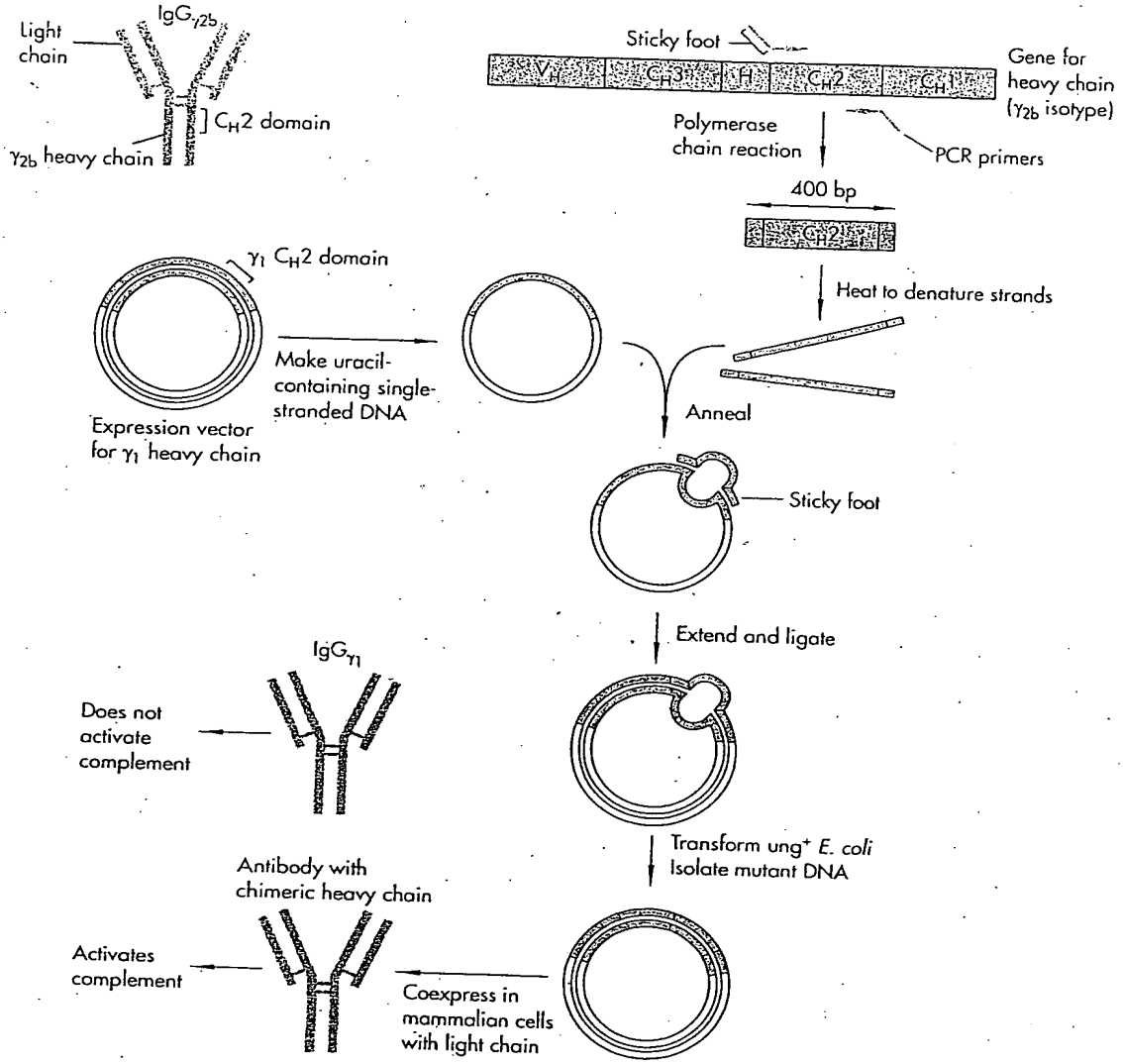


FIGURE 11-15

Construction of a chimeric antibody heavy-chain-encoding gene by "sticky feet–directed" mutagenesis. Antibodies containing a γ2b heavy chain are known to participate in complement-dependent cell lysis, whereas antibodies containing γ1 heavy chains do not. In order to identify which domain of the γ2b heavy chain is responsible for this property, an antibody containing a chimeric heavy chain was produced. To construct a gene encoding the chimeric heavy chain, a 400-bp fragment encoding the C_H2 domain from a γ1 heavy chain was replaced with the homologous segment from a γ2b gene. Since there were no convenient restriction sites at the ends of the C_H2 segments, the 400-nucleotide-long γ2b DNA was prepared by PCR. The PCR primers were complementary to the ends of the γ2b DNA but contained additional nucleotides (the sticky feet) that were complementary to γ1 DNA at the boundaries of the γ1 C_H2 domain. The strands of the PCR-generated fragment were separated by heating, then one strand was used as the primer in a mutagenesis experiment using a uracil-containing single-stranded γ1 DNA template by the method shown in Figure 11-10. The resulting chimeric heavy-chain gene was coexpressed with a light chain gene in mammalian cells to form an antibody that now activated complement. Since only the C_H2 domain came from the γ2b heavy-chain, this result demonstrated that the γ2b C_H2 domain contains the information necessary to activate complement-dependent cell lysis. Sticky feet–directed mutagenesis provided a simple means for constructing this complicated gene.

that helps to narrow down important amino acids in a protein is the analysis of chimeras between related proteins. We have previously discussed the use of computer programs to identify related proteins by comparison of their amino acid sequences (Chapter 8). Chimeric proteins are constructed by replacing a segment of one protein with the *homologous* segment from another protein. Although the two proteins have functional differences, their sequence similarity often indicates that they share a common overall structure. A striking example of this was in the analysis of human growth hormone (hGH). A series of chimeric proteins were made in which most of the amino acids were derived from hGH but which contained segments from related hormones, such as human prolactin. Using this strategy, regions of hGH that interact with the hGH receptor were identified. In Chapter 17, we will see how functional regions of a receptor which spans the membrane seven times were identified by the study of chimeras.

The 434/P22 repressor (Figure 11-12) and hGH chimeras were constructed by ligation of short oligonucleotide cassettes into the coding sequence. A different strategy (Figure 11-15) was used to prepare a chimeric antibody in which a 400-bp segment from a $\gamma 1$ heavy-chain gene was replaced by the homologous segment from a $\gamma 2b$ gene. A 400-bp DNA fragment was generated by PCR that encoded the new sequence to be inserted and two 30-base "sticky feet" on each end. The double-stranded PCR fragment was heated to denature the two strands, and then one of the single-stranded molecules was utilized in a primer-extension experiment (as in Figure 11-8). Had the gene synthesis method been employed, construction of the chimeric gene would have required twenty 40-nucleotide-long oligomers. Instead, the sticky feet method used only two oligonucleotide primers for PCR.

Mutagenesis Is the Gateway to Gene Function and Protein Engineering

It would be difficult to overestimate the importance of in vitro mutagenesis techniques to biology and biotechnology. The harnessing of enzymes that operate on DNA and the refinement of oligonucleotide synthesis have made changing gene sequences an almost trivial task. And the ability to operate on DNA lets us also change the structure of the products of genes—RNA and, most importantly, proteins. Thus, the impact of this technology is twofold. It has revolutionized how research is done in molecular biology by creating the entirely new concept of "reverse genetics"—changing gene sequence first, then examining gene function. And it opens the door to sophisticated protein engineering (see Chapter 23), the ability to make changes in natural gene products that make them do their jobs better. The impact of protein engineering on medicine and industry will be substantial.

Reading List

General

Wu, R., L. Grossman, and K. Moldave, eds. *Methods in Enzymology*, vol. 100: *Recombinant DNA, Part B.* Academic, New York, 1983.

Gait, M. *Oligonucleotide Synthesis: A Practical Approach.* IRL, Oxford, Eng., 1984.

Wu, R., and L. Grossman, eds. *Methods in Enzymology*, vol 154: *Recombinant DNA, Part E. Academic*, New York, 1987.

Original Research Papers

STRATEGIES FOR IN VITRO MUTAGENESIS

Botstein, D., and D. Shortle. "Strategies and applications of in vitro mutagenesis." *Science*, 229: 1193–1201 (1985). [Review]

Smith, M. "In vitro mutagenesis." *Ann. Rev. Gen.*, 19: 423–462 (1985). [Review]

Zoller, M. J. "New molecular biology methods for protein engineering." *Curr. Opin. Struct. Biol.*, 1: 605–610 (1991). [Review]

INSERTION MUTAGENESIS

Heffron, F., M. So, and B. J. McCarthy. "In vitro mutagenesis of a circular DNA molecule using synthetic restriction sites." *Proc. Natl. Acad. Sci. USA*, 75: 6012–6016 (1978).

Abraham, J., J. Feldman, K. A. Nasmyth, J. N. Strathern, A. J. S. Klar, J. R. Broach, and J. B. Hicks. "Sites required for position-effect regulation of mating type information in yeast." *Cold Spring Harbor Symp. Quant. Biol.*, 47: 989–998 (1983).

Rees-Jones, R. W., and S. P. Goff. "Insertional mutagenesis of the Abelson murine leukemia virus genome: identification of mutants with altered kinase activity and defective transformation ability." *J. Virol.*, 62: 978–986 (1988).

DELETION MUTAGENESIS

Lai, C. J., and D. Nathans. "Deletion mutants of SV40 generated by enzymatic excision of DNA segments from the viral genome." *J. Mol. Biol.*, 89: 179–193 (1974).

Mertz, J. E., J. Carbon, M. Herzberg, R. W. Davis, and P. Berg. "Isolation and characterization of individual clones of simian virus 40 mutants containing deletions, duplications and insertions in their DNA." *Cold Spring Harbor Symp. Quant. Biol.*, 39: 69–84 (1974).

Sakonju, S., D. Bogenhagen, and D. D. Brown. "A control region in the center of the 5S gene directs specific initiation of transcription, I: the 5′ border of the region." *Cell*, 19: 13–26 (1980).

McKnight, S. L., and R. Kingsbury. "Transcriptional control signals of a eukaryotic protein-coding gene." *Science*, 217: 316–324 (1982).

Struhl, K. "The yeast his3 promoter contains at least two distinct elements." *Proc. Natl. Acad. Sci. USA*, 79: 7385–7389 (1982).

CHEMICAL MUTAGENESIS

Chu, C. T., D. S. Parris, R. A. F. Dixon, F. E. Farber, and P. A. Schaffer. "Hydroxylamine mutagenesis of HSV DNA and DNA fragments: introduction of mutations into selected regions of the viral genome." *Virol.*, 98: 168–181 (1979).

Shortle, D., and D. Nathans. "Regulatory mutants of simian virus 40: constructed mutants with base substitutions at the origin of viral DNA replication." *J. Mol. Biol.*, 131: 801–817 (1979).

Weiher, H., and H. Schaller. "Segment-specific mutagenesis: extensive mutagenesis of a lac promoter/operator element." *Proc. Natl. Acad. Sci. USA*, 79: 1408–1412 (1982).

Myers, R. M., L. S. Lerman, and T. Maniatis. "A general method for saturation mutagenesis of cloned DNA fragments." *Science*, 229: 242–247 (1985).

ENZYMATIC MISINCORPORATION MUTAGENESIS

Shortle, D. P. Grisafi, S. J. Benkovic, and D. Botstein. "Gap misrepair mutagenesis: efficient site-directed introduction of transition, transversion, and frameshift mutations in vitro." *Proc. Natl. Acad. Sci. USA*, 79: 1588–1592 (1982).

Zakour, R. A., and L. A. Loeb. "Site-specific mutagenesis by error-directed DNA synthesis." *Nature*, 295: 708–710 (1982).

Abarzua, P., and K. J. Marians. "Enzymatic techniques for the isolation of random single-base substitutions in vitro at high frequency." *Proc. Natl. Acad. Sci. USA*, 81: 2030–2034 (1984).

Leung, D. W., E. Chen, and D. V. Goeddel. "A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction." *Technique*, 1: 11–15 (1989)

OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS

Hutchinson, C. A., Phillips, M. H. Edgell, S. Gillam, P. Jahnke, and M. Smith. "Mutagenesis at a specific position in a DNA sequence." *J. Biol. Chem.*, 253: 6551–6560 (1978).

Wallace, R. B., M. Schold, M. J. Johnson, P. Dembek, and K. Itakura. "Oligonucleotide-directed mutagenesis of the human β-globin gene: a general method for producing specific point mutations in cloned DNA." *Nuc. Acids Res.*, 9: 3642–3656 (1981).

Zoller, M. J., and M. Smith. "Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for production of point mutations in any fragment of DNA." *Nuc. Acids Res.*, 10: 6487–6500 (1982).

Kramer, W., V. Drutsa, H. W. Jansen, M. Pflugfelder, and H.-J. Fritz. "The gapped duplex DNA approach to oligonucleotide-directed mutation construction." *Nuc. Acids Res.*, 12: 9441–9456 (1984).

Kunkel, T. A. "Rapid and efficient site-specific mutagenesis without phenotypic selection." *Proc. Natl. Acad. Sci. USA*, 82: 477–492 (1985).

Taylor, J. W., J. Ott, and F. Eckstein. "The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA." *Nuc. Acids Res.*, 13: 8765–8785 (1985).

CASSETTE MUTAGENESIS

Lo, K.-M., S. S. Jones, N. R. Hackett, and H. G. Khorana. "Specific amino acid substitutions in bacterioopsin: replacement of a restriction fragment in the structural gene by synthetic DNA fragments containing altered codons." *Proc. Natl. Acad. Sci. USA*, 81: 2285–2289 (1984).

Wells, J. A., M. Vasser, and D. B. Powers. "Cassette mutagenesis: an efficient method for generation of multiple mutations at defined sites." *Gene*, 34: 315–323 (1985).

Wharton, R., and M. Ptashne. "Changing the binding specificity of a repressor by redesigning an alpha-helix." *Nature*, 316: 601–605 (1985).

Reidhaar-Olson, J. F., and R. T. Sauer. "Combinatorial cassette mutagenesis as a probe of the informational content of protein sequences." *Science*, 241: 53–57 (1988).

DOPED OLIGONUCLEOTIDE MUTAGENESIS

McNeil, J. B., and M. Smith. "*Saccharomyces cerevisiae* CYC1 mRNA 5'-end positioning: analysis by in vitro mutagenesis, using synthetic duplexes with random mismatch base pair." *Mol. Cell. Biol.*, 5: 3545–3551 (1985).

Hutchison, C. A., S. K. Nordeen, K. Vogt, and M. H. Edgell. "A complete library of point substitution mutations in the glucocorticoid response element of mouse mammary tumor virus." *Proc. Natl. Acad. Sci. USA*, 83: 710–714 (1986).

GENE SYNTHESIS

Ferreti, L., S. S. Karnik, H. G. Khorana, N. Nassal, and D. D. Oprian. "Total synthesis of a gene for bovine rhodopsin." *Proc. Natl. Acad. Sci. USA*, 83: 599–603 (1986).

Caruthers, M. H., A. D. Barone, S. L. Beaucage, D. R. Dodds, E. F. Fisher, L. J. McBride, M. Matteucci, Z. Stabinsky, and J.-Y. Tang. "Chemical synthesis of deoxyoligonucleotides by the phosphoramidite method." *Meth. Enzymol.*, 154: 287–313 (1987).

Abate, C., D. Luk, R. Getz, F. J. Rauscher, III, and T. Curran. "Expression and purification of the leucine zipper and DNA-binding domains of Fos and Jun: both Fos and Jun contact DNA directly." *Proc. Natl. Acad. Sci. USA*, 87: 1032–1036 (1990).

CONSTRUCTING CHIMERIC GENES BY PCR

Higuchi, R., B. Krummel, and R. K. Saiki. "A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions." *Nuc. Acids Res.*, 16: 7351–7367 (1988).

Clackson, T., and G. Winter. "'Sticky feet'-directed mutagenesis and its application to swapping antibody domains." *Nuc. Acids Res.*, 17: 10163–10170 (1989).

PROTEIN STRUCTURE AND FUNCTION

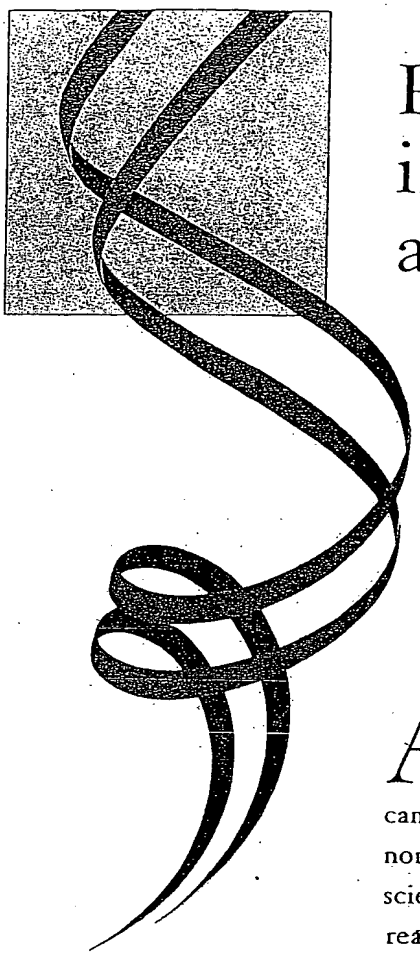
Winter, G., A. R. Fersht, A. J. Wilkinson, M. Zoller, and M. Smith. "Redesigning enzyme structure by site-directed mutagenesis." *Nature*, 299: 756–758 (1982).

Cunningham, B. C., P. Jhurani, P. Ng, and J. A. Wells. "Receptor and antibody epitopes in human growth hormone identified by homolog-scanning mutagenesis." *Science*, 243: 1330–1336 (1989).

Gibbs, C. S., and M. J. Zoller. "Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalytic and substrate interactions." *J. Biol. Chem.*, 266: 8923–8931 (1991).

CHAPTER 23

Recombinant DNA in Medicine and Industry



As soon as the first successful cloning experiments were reported in 1973, applications for this powerful technology quickly followed. The significance of being able to produce large quantities of human proteins that were normally available in exceedingly small amounts, if at all, was not lost on scientists, physicians, and businessmen alike. In 1976 *biotechnology* became a reality as the methodologies for DNA cloning, oligonucleotide synthesis, and gene expression converged in a single experiment, in which a human protein was expressed from recombinant DNA for the first time. The protein was somatostatin, a 14 amino acid peptide neurotransmitter. The gene encoding somatostatin was not the natural gene but was synthesized chemically and cloned into a plasmid vector for expression in *E. coli*. Soon after followed the successful expression of human insulin for the treatment of diabetes, the first commercial product of the biotechnology industry. Instead of insulin extracted from the pancreases of pigs and cows, diabetics could now receive insulin identical to that normally produced by humans.

The ability to achieve such feats relied on the successes in all areas of molecular biology, including oligonucleotide synthesis, isolation of enzymes that cleave and join DNA, characterization of bacterial plasmids, and an understanding of gene expression. These methods have, of course, revolutionized research in

biology and medicine, but what is equally important, they have spawned an entirely new industry, one devoted to the cloning and production of proteins of importance to both medicine and industry. Today, proteins are produced through recombinant DNA technology for treatment of numerous diseases—cancer, allergies, autoimmune disease, neurological disorders, heart attacks, blood disorders, infections, wounds, and genetic diseases—as well as for more prosaic tasks, such as use in laundry detergents and food production. In addition, entirely new approaches to drug design have emerged from recombinant DNA technology, as scientists have gained the ability to tinker with natural proteins to improve their function and to change them in subtle and useful ways.

Expression Systems Are Developed to Produce Recombinant Proteins

Cloning the gene or cDNA encoding a particular protein is only the first of many steps needed to produce a recombinant protein for medical or industrial use. The next step is to put the gene into a host cell for production. The development of expression systems has been an important research area in both industrial and academic laboratories. The most popular expression systems are the bacteria *E. coli* and *Bacillus subtilis*, yeast, and cultured insect and mammalian cells. We have learned in earlier chapters about the development of vectors and DNA transformation methods for these organisms. Here we will discuss the issues that are important for protein production. The choice of which cell is used depends on the project goals and on the properties of the protein to be produced.

Bacterial cells offer simplicity, short generation times, and large yields of product with low costs. And, especially with *B. subtilis*, the cells can be induced to secrete the product into the culture medium, thus greatly simplifying the task of purification. But expression in prokaryotic cells has several drawbacks. Although some proteins are expressed to high levels (greater than 10 percent of the mass of all bacterial proteins), they often fail to fold properly and hence form insoluble *inclusion bodies*. Protein extracted from these inclusion bodies is often biologically inactive. Small proteins can sometimes be refolded into their active forms, but larger proteins usually cannot. A second problem is that foreign proteins are sometimes toxic to bacteria, so cell cultures producing the protein cannot be grown to high densities. This problem can often be circumvented by using an inducible promoter that is turned on to begin transcription of the gene for the foreign protein only after the culture has been grown. Third, bacterial cells lack enzymes that are present in eukaryotic cells and add posttranslational modifications, such as phosphates and sugars, to proteins. These modifications are often required for proper functioning of proteins. Researchers are addressing this problem by purifying the eukaryotic enzymes that carry out these modifications and using these enzymes to add the needed modifications to bacterially expressed proteins.

Yeast has been used for centuries by brewers and bakers, and now it toils for biotechnologists as well. As discussed in Chapter 13, yeast is a simple eukaryote that resembles mammalian cells in many ways but can be grown as quickly and cheaply as bacteria can. Yeast perform many of the posttranslational modifications found on human proteins and can be induced to secrete certain proteins into the growth medium for harvesting. A disadvantage of yeast is the presence of active proteases that degrade foreign proteins, thereby reducing the yield of product. Researchers are dealing with this problem, however, by constructing yeast strains in which the protease genes have been deleted.

Expression of heterologous proteins in insect cells by baculovirus vectors (as previously described in Figure 12-12) is a relatively new approach. The main advantages are high-level expression, correct folding, and posttranslational modifications similar to those in mammalian cells. A vaccine for the AIDS virus has been prepared by producing one of the HIV glycoproteins with this system. Although the cost of culturing insect cells is currently more than that for culturing bacteria and yeast, it is less than that for culturing mammalian cells.

Despite the significant advantages of producing human proteins in heterologous host cells, in some cases the best place to produce a mammalian protein is in mammalian cells. Great improvements have been made to promoters, vectors, transformation protocols, and host cell systems. Transient expression in mam-

malian cells (described in Figure 12-4) is often used for checking the function of a newly cloned gene and as a quick method for assessing the function of engineered proteins. The extracellular domains of cell-surface receptors (Chapter 17) have been engineered for secretion from cells by introducing a stop codon into the gene before the transmembrane domain sequence. These *soluble receptors* are valuable reagents for studying ligand binding in vitro and for screening for receptor agonists or antagonists, and they may eventually be used as therapeutics themselves. Although transient systems yield enough protein for laboratory experiments, stably integrated amplified genes in mammalian cells are used for the large-scale production of proteins such as tissue plasminogen activator, which we describe later.

Insulin Is the First Recombinant Drug Licensed for Human Use

The first licensed drug produced through genetic engineering was human insulin. An important hormone that regulates sugar metabolism, insulin is produced by a small number of cells in the pancreas and secreted into the bloodstream. An inability to produce insulin results in diabetes, but daily injections of insulin are sufficient to reverse or at least allay the debilitating effects of the disease. Prior to production of the recombinant molecule, insulin for treatment of diabetes was obtained from the pancreases of pigs and cows. Although this insulin is biologically active in humans, the amino acid sequences are not identical to that of the human molecule. Thus, some patients produced antibodies against injected insulin, occasionally resulting in serious immune reactions. Because recombinant human insulin is identical to the natural product, immunogenicity should not be a problem.

In mammals, insulin is expressed as a single-chain *prepro-hormone,* which is secreted through the plasma membrane. A prepro-hormone contains extra amino acids not present in the mature hormone. Amino-terminal amino acids form the *pre* sequence and target the expressed protein for secretion. The *pro* sequence is a stretch of amino acids in the middle of the hormone sequence that is important for folding the polypeptide chain into the correct structure. During secretion, these extra amino acids are cleaved from the prepro-hormone by cellular proteases to release the mature insulin molecule, consisting of two short polypeptide chains, A and B, linked by two disulfide bonds. The principal challenge in the production of recombinant insulin was getting insulin assembled into this mature form. The initial approach was to construct synthetic genes from oligonucleotides that separately encoded the A and B chains. These were individually inserted into the *E. coli* gene encoding β-galactosidase, so the bacteria produced large fusion proteins that had the insulin sequences tacked onto the end of the β-galactosidase enzyme (Figure 23-1). These large proteins were purified from bacterial extracts, and the insulin chains were released by treatment with cyanogen bromide, a chemical that cleaves peptide bonds following methionine residues. Because a methionine codon had been inserted at the boundaries between β-galactosidase and the insulin chains in the fusion proteins, cyanogen bromide treatment clipped intact insulin chains off the fusion proteins. These were purified, mixed, and reconstituted into an active insulin molecule. This approach was refined by producing a single β-galactosidase–insulin fusion protein, which could be cleaved in a single step to release mature insulin. A similar method is now in use for the commercial production of recombinant insulin.

Recombinant Human Growth Hormone is Produced in Bacteria by Two Methods

Growth hormone is a 191 amino acid protein that is produced in the pituitary gland and regulates growth and development. Children born with growth hormone deficiency—hypopituitary dwarfs—never achieve normal stature. Regular injections of growth hormone stimulate the growth of these children so that they reach near-normal heights. Unlike the situation with insulin, animal-derived growth hormones are ineffective. Only the human protein works, and for many years it was painstakingly extracted from the pituitaries of human cadavers. One unforeseen and

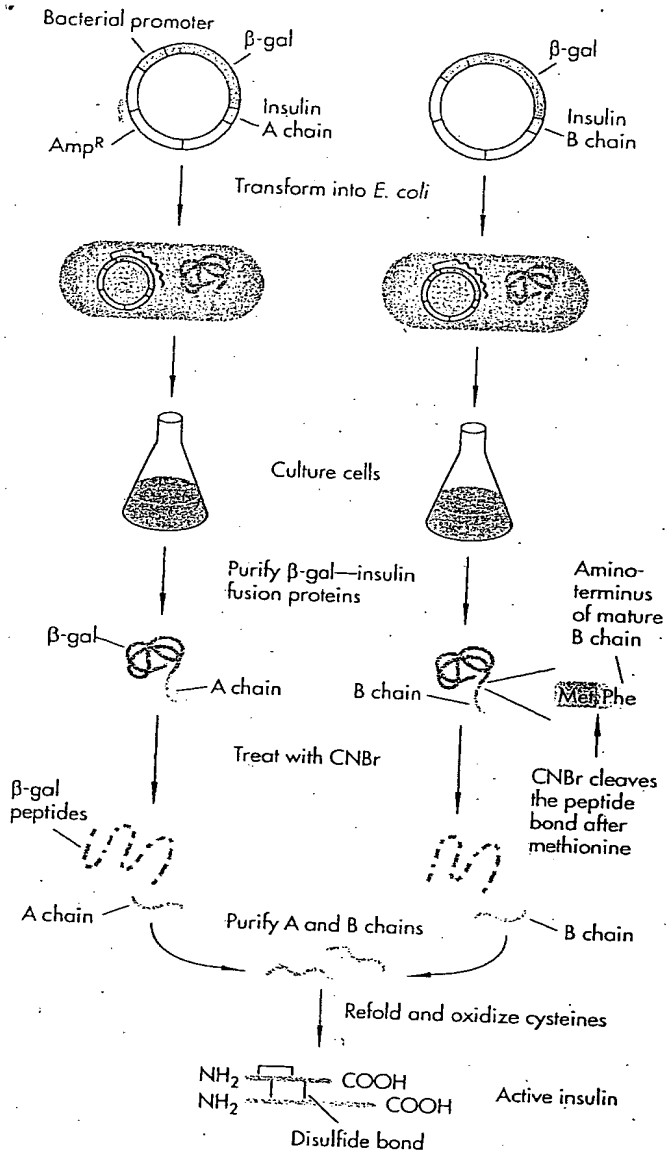


FIGURE 23-1
Expression of human insulin in *E. coli*. Recombinant insulin was first made by expressing the A and B chains separately, then refolding them into a mature insulin molecule. A DNA fragment encoding each insulin chain was made by annealing two complementary oligonucleotides that had been chemically synthesized. Each fragment was ligated into a bacterial expression vector so that, when translated, the insulin chain would be fused to the carboxy terminus of the enzyme β-galactosidase (β-gal). The expression vectors were transformed into *E. coli*, and the β-gal–insulin fusion proteins accumulated inside the bacterial cells. The cells were harvested, and each β-gal–insulin fusion protein was purified. The insulin-coding DNA was synthesized so that it started with a methionine codon. This setup provided a way to cleave off the β-gal part from the insulin polypeptide. Treatment of the fusion protein with the chemical cyanogen bromide (CNBr) results in cleavage of peptide bonds after all methionines. In this way, the natural insulin peptides were obtained. Because β-gal contains other methionine residues, CNBr treatment cleaved it into many small peptides. The insulin chains were not cleaved further because they did not contain internal methionines. The A and B chains were purified and then mixed together to form active recombinant insulin.

unfortunate consequence of growth hormone treatment, however, was the infection of a number of children with a fatal virus from one of the cadavers. Production of recombinant human growth hormone (hGH) would clearly provide a safe, reliable, and plentiful source of this drug.

The initial production of hGH was achieved by constructing a hybrid gene from the natural hGH cDNA and synthetic oligonucleotides that encoded the amino terminus of the mature form of the protein (Figure 23-2a). This coding sequence was ligated into a plasmid adjacent to a bacterial promoter. Like insulin, hGH is normally produced as a larger precursor protein containing an amino-terminal signal sequence. Because the human signal sequence would not be recognized by the bacterial secretion machinery, the 5′ end of the cDNA was reengineered with a synthetic DNA sequence enabling the bacteria to produce a nearly normal version of the mature human protein.

The first hGH expression vectors directed the production of the protein inside the cell. Purification required many steps to separate hGH from the thousands of intracellular bacterial proteins. Another way to produce the protein in bacteria is to engineer the protein so it is secreted. This can be done by linking the coding sequence for the desired protein to a signal sequence from a secreted bacterial protein, thus forming a *prehormone* (Figure 23-2b). Human growth hormone is produced by the bacteria and then secreted with the concomitant removal of the signal peptide by a bacterial protease. Secretion into the periplasm, where there are fewer proteins than inside the cell, makes purification simpler. The only difference between the secreted hGH and that produced intracellularly is the presence of an amino-terminal methionine on the intracellularly expressed molecule. Because the secreted

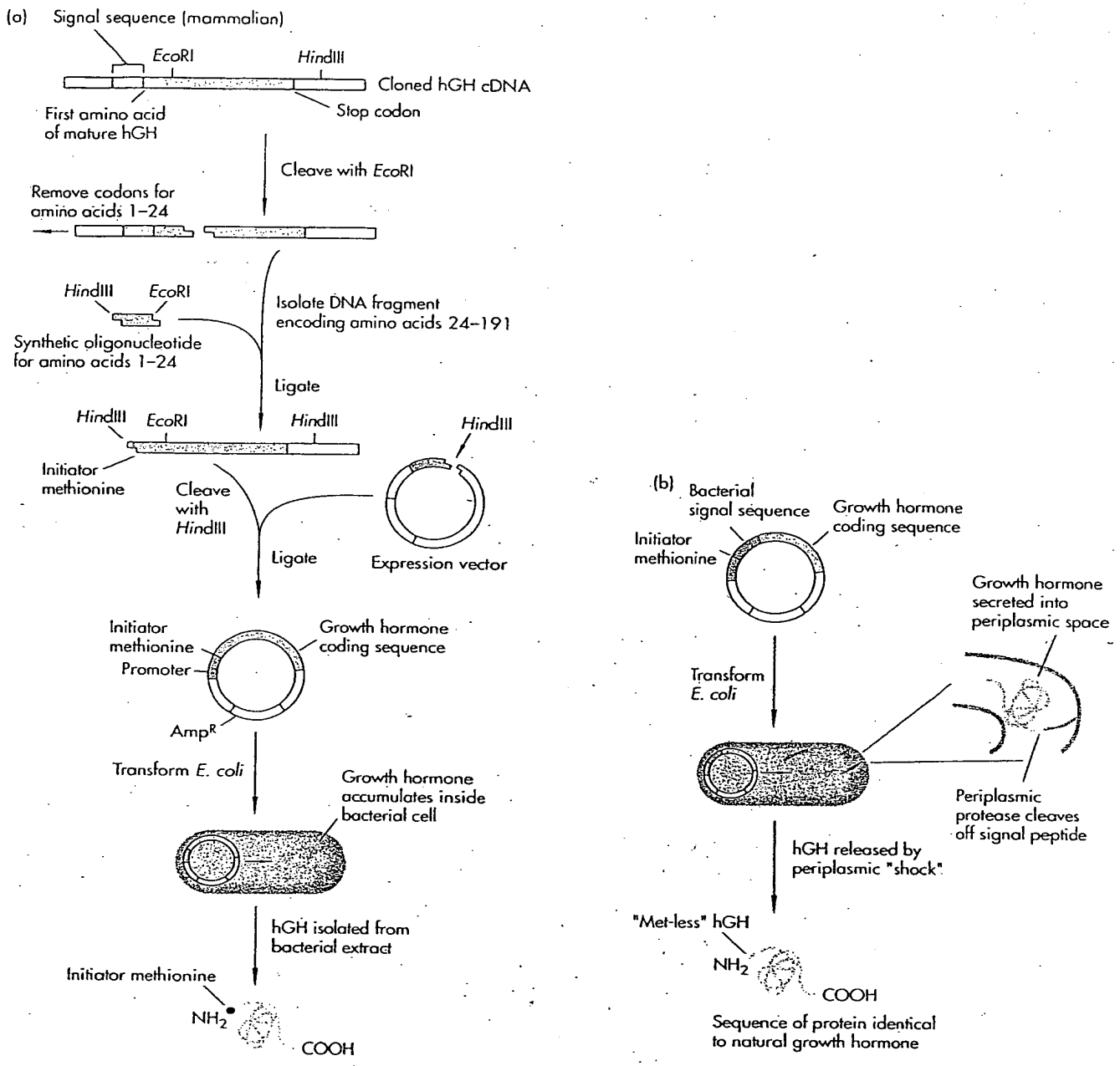


FIGURE 23-2

Bacterial production of human growth hormone (hGH). (a) An expression vector was constructed for intracellular production of hGH. The coding sequence was constructed by isolating from the cDNA a DNA fragment that encoded amino acids 24–191 and ligating this to a synthetic oligonucleotide fragment that encoded amino acids 1–24. Following introduction of the expression vector into bacterial cells, recombinant hGH was produced inside the cells. The expressed protein behaved just like natural human growth hormone but contained the initiator methionine at the amino terminus. (b) A protein can be produced in bacteria without this extra methionine by targeting it for secretion. To do this, a DNA fragment encoding a bacterial *signal sequence*, which specifies secretion of a bacterial protein, was placed in front of the hGH coding sequence. Upon introduction of this vector into bacteria, hGH is produced, and the signal sequence targets the protein for secretion. The protein accumulates in the *periplasmic space* between the inner and outer bacterial membranes and can be released by hypotonic disruption of the outer membrane. In contrast to the intracellular form of hGH, the protein produced by this procedure does not contain an initiator methionine, since a periplasmic protease cleaved off the signal sequence.

form lacks this methionine, it is called *met-less* hGH. Bacterially expressed hGH has been administered to thousands of growth hormone–deficient children, who have benefited greatly from this recombinant drug.

A Hepatitis B Virus Vaccine Is Produced in Yeast by Expression of a Viral Surface Antigen

One of the successes of modern medicine is the development and implementation of vaccines against infectious diseases. Prior to the advent of recombinant DNA technology, two types of vaccines were used. *Inactivated* vaccines are chemically killed derivatives of the actual infectious agent. *Attenuated* vaccines are live viruses or bacteria altered so that they no longer multiply in the inoculated organism. Both types of vaccines work by presenting surface proteins (antigens) to B and T lymphocytes, which become primed to respond rapidly should the organism actually become infected, usually destroying the infectious agent before any damage is done (Chapter 16). However, these types of vaccines are potentially dangerous because they can be contaminated with infectious organisms. For example, a small number of children each year contract polio from their polio vaccinations. Thus, one of the most promising applications of recombinant DNA technology is the production of *subunit vaccines*, consisting solely of the surface protein to which the immune system responds. With a subunit vaccine, there is no risk of infection.

The first successful subunit vaccine was produced for hepatitis B virus (HBV), which infects the liver and causes liver damage and, in some cases, cancer. The virus particle is coated with a surface antigen, HBsAg, and infected patients carry large aggregates of this protein in their blood. Early experiments suggested that these aggregates would make a potent vaccine, but how could they be produced in quantities sufficient to vaccinate large populations against HBV? With the cloning of the HBV genome, the possibility of a subunit vaccine could be explored. Initial attempts to produce the HBsAg protein in *E. coli* failed, so researchers turned to yeast. The HBsAg gene was inserted into a high-copy yeast expression vector (Figure 13-3) and engineered, in this case, so that it would not be secreted (Figure 23-3). Yeast transformed with this plasmid produced large quantities of the viral protein (about 1–2 percent of the total yeast protein). By growing the yeast in large fermentors, it was possible to produce 50–100 mg of the protein per liter of culture. This recombinant protein closely resembled the natural viral protein; it even formed aggregates with properties similar to those of the immunogenic aggregates found in infected patients. The yeast protein is now used commercially to vaccinate people against HBV infection.

Vaccines against many human and animal pathogens are currently in various stages of development. Recombinant DNA technology has provided a safe means to work with and to inoculate children and adults with only noninfectious parts of infectious agents. In Chapter 25, we will discuss various strategies for the development of a vaccine against the AIDS virus.

Complex Human Proteins Are Produced by Large-Scale Mammalian Cell Culture

Most of the recombinant proteins we have discussed thus far in this chapter are relatively small and simple in both structure and function. Other proteins of medical interest are considerably more complicated in structure and function, and biologically active proteins have proved difficult to produce in bacteria and yeast. In these cases, biotechnology companies have resorted to using mammalian cells for protein production. Mammalian cells are finicky and expensive to grow, but they can be counted on to produce correctly modified, fully active proteins. Thus, much effort in the biotechnology industry has been devoted to setting up fermentor systems for large-scale culture of mammalian cells.

The first drug to be produced commercially by mammalian cell culture was *tissue plasminogen activator* or *tPA*, which is administered to heart attack victims. Tissue plasminogen activator is a protease, an enzyme that cleaves other proteins. It works by clipping *plasminogen*, an inactive precursor protein, to form *plasmin*, itself a potent protease that degrades *fibrin*, the protein

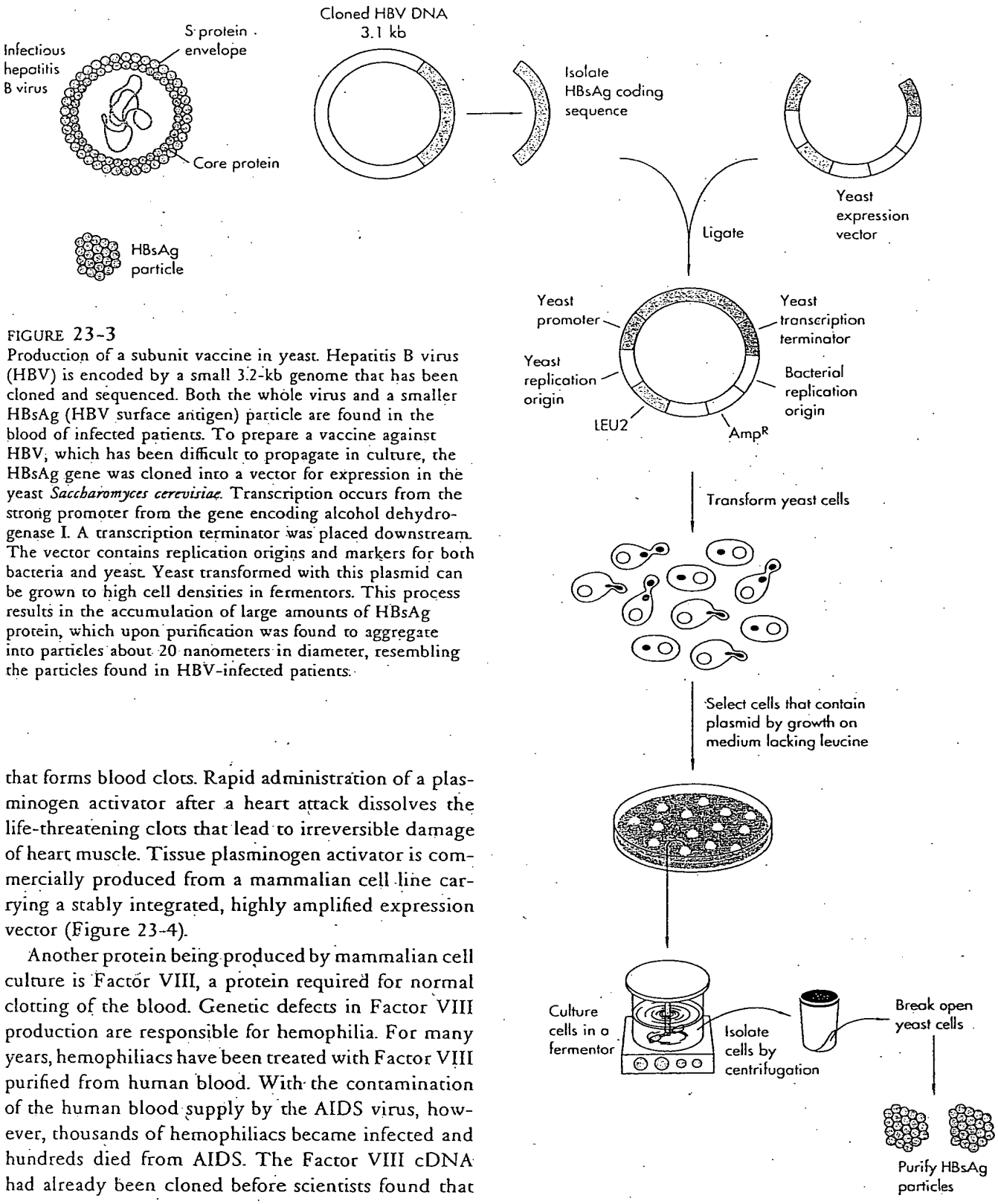


FIGURE 23-3
Production of a subunit vaccine in yeast. Hepatitis B virus (HBV) is encoded by a small 3.2-kb genome that has been cloned and sequenced. Both the whole virus and a smaller HBsAg (HBV surface antigen) particle are found in the blood of infected patients. To prepare a vaccine against HBV, which has been difficult to propagate in culture, the HBsAg gene was cloned into a vector for expression in the yeast *Saccharomyces cerevisiae*. Transcription occurs from the strong promoter from the gene encoding alcohol dehydrogenase I. A transcription terminator was placed downstream. The vector contains replication origins and markers for both bacteria and yeast. Yeast transformed with this plasmid can be grown to high cell densities in fermentors. This process results in the accumulation of large amounts of HBsAg protein, which upon purification was found to aggregate into particles about 20 nanometers in diameter, resembling the particles found in HBV-infected patients.

that forms blood clots. Rapid administration of a plasminogen activator after a heart attack dissolves the life-threatening clots that lead to irreversible damage of heart muscle. Tissue plasminogen activator is commercially produced from a mammalian cell line carrying a stably integrated, highly amplified expression vector (Figure 23-4).

Another protein being produced by mammalian cell culture is Factor VIII, a protein required for normal clotting of the blood. Genetic defects in Factor VIII production are responsible for hemophilia. For many years, hemophiliacs have been treated with Factor VIII purified from human blood. With the contamination of the human blood supply by the AIDS virus, however, thousands of hemophiliacs became infected and hundreds died from AIDS. The Factor VIII cDNA had already been cloned before scientists found that

the blood supply was contaminated with the AIDS virus. Recognition of the need for a safer source of Factor VIII accelerated efforts already under way to produce the protein using recombinant DNA methods. Like tPA, Factor VIII is a large and complex protein and can only be efficiently produced in mammalian cell culture. But the availability of recombinant protein will spare future generations of hemophiliacs from infectious agents that contaminate the blood supply.

Monoclonal Antibodies Function as "Magic Bullets"

We have discussed the use of biotechnology to produce novel vaccines that elicit antibody production by the body's immune system. As we learned in Chapter 16, antibodies are exquisitely selective proteins that can bind to a single target among millions of irrelevant sites. Researchers have long dreamed of harnessing the specificity of antibodies for a variety of uses that require the targeting of drugs and other treatments to particular sites in the body. It is this use of antibodies as targeting devices that led to the concept of the "magic bullet," a treatment that could effectively seek and destroy tumor cells and infectious agents wherever they resided.

The major limitation in the therapeutic use of antibodies is producing a useful antibody in large quantities. Initially, researchers screened *myelomas*, which are antibody-secreting tumors, for the production of

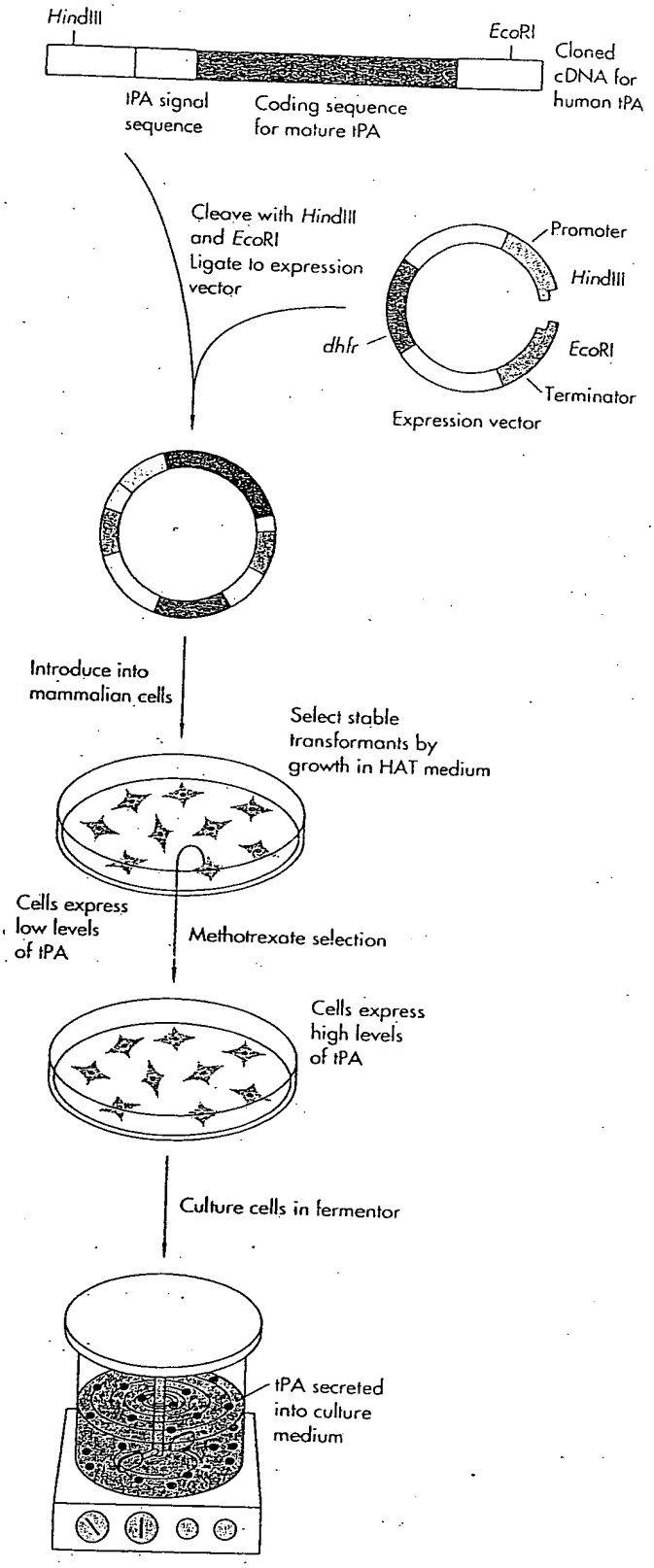


FIGURE 23-4
Production of tissue plasminogen activator (tPA) by mammalian cell culture. The cloned cDNA for human tPA was ligated into an expression vector that contained a strong promoter and terminator. The vector was stably transfected into a mammalian cell line. The initial transformants secreted tPA into the culture medium, but the level of expression was very low. Cell lines that expressed tPA to high levels were obtained using methotrexate treatment, which selects for cells that have amplified the *dhfr* gene resident in the vector together with the linked tPA expression cassette (Chapter 12). High-expressing lines are grown in large fermentors and recombinant tPA is purified from the culture medium.

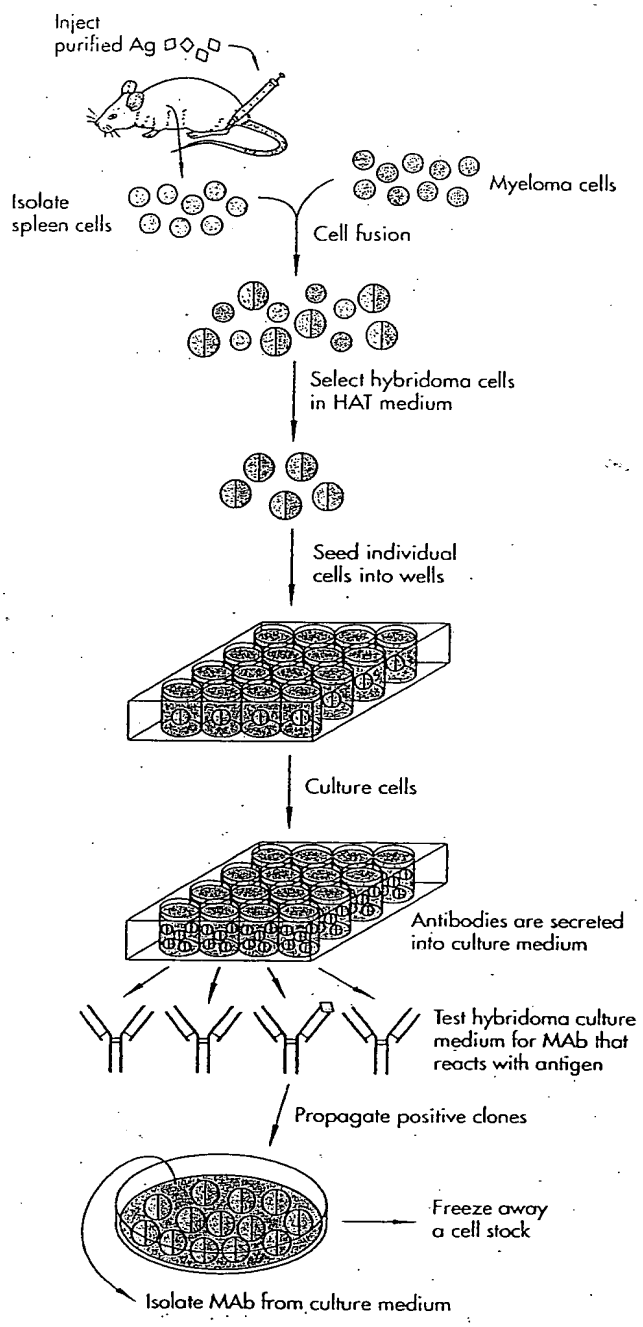


FIGURE 23-5
Production of a monoclonal antibody (MAb). A mouse is inoculated with an antigen (Ag) of interest. This stimulates the proliferation of lymphocytes expressing antibodies against the antigen. Lymphocytes are taken from the spleen and fused to myeloma cells by treatment with polyethylene glycol. Hybrid cells are selected by growth in HAT medium (Chapter 12). The myeloma cells lack the enzyme HPRT and thus die in this medium unless they become fused with a lymphocyte, which expresses the missing enzyme. Unfused lymphocyte cells soon die off as well, because they do not grow for long in culture. Individual hybrid cells are transferred to the wells of a microtiter dish and cultured for several days. Aliquots of the culture fluids are removed and tested for the presence of antibody (Ab) that binds the antigen. Cells that test positive are cultured for monoclonal antibody production. Antibody-producing cell lines are stored frozen in liquid nitrogen (this process is called *cell banking*). Aliquots can be thawed out and cultured as needed.

useful antibodies. But they lacked a means to program a myeloma to produce an antibody to their specifications. This situation changed dramatically with the development of *monoclonal antibody* technology. The procedure for producing monoclonal antibodies, or MAbs, is shown in Figure 23-5. First, a mouse or rat is inoculated with the antigen to which an antibody is desired. After the animal mounts an immune response to the antigen, its spleen, which houses antibody-producing cells (lymphocytes), is removed, and the spleen cells are fused en masse to a specialized myeloma cell line that no longer produces an antibody of its own. The resulting fused cells, or *hybridomas*, retain properties of both parents. They grow continuously and rapidly in culture like the myeloma cell, yet they produce antibodies specified by the lymphocyte from the immunized animal. Hundreds of hybridomas can be produced from a single fusion experiment, and they are systematically screened to identify those producing large amounts of a desired antibody. Once identified, this antibody is available in limitless quantities. Monoclonal antibodies are already widely used for the diagnosis of infections and cancer and for the imaging of tumors for radiotherapy. And investigations into their use in the direct treatment of cancer, inflammation, and immune disorders is on the rise.

Human Antibodies That Recognize Specific Antigens Can Be Directly Cloned and Selected

One new application of monoclonal antibody technology is the generation of *abzymes*, antibodies that behave like enzymes to catalyze a chemical reaction.

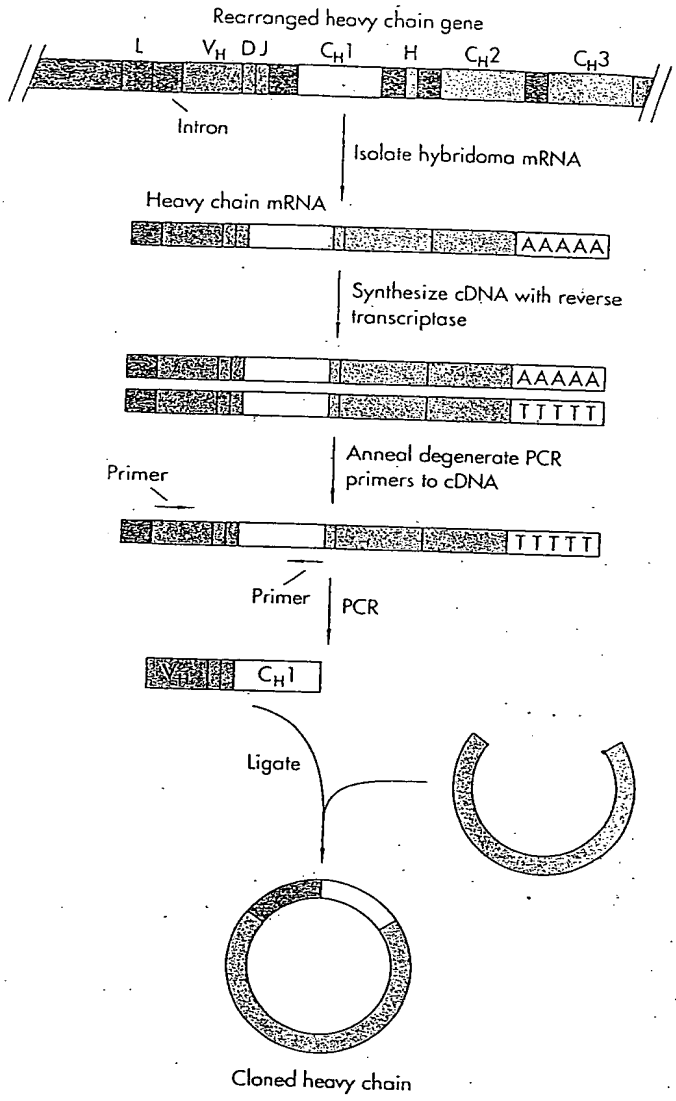


FIGURE 23-6
Direct cloning of antibody cDNAs by PCR. To engineer an antibody, the amino acid sequence of the variable domain needs to be determined. This could be done by sequencing a purified preparation of the heavy- (H) and light- (L) chain proteins, but a simpler method is to deduce the sequence from the cloned cDNA. In the past, a cDNA library was prepared from hybridoma mRNA and screened with probes from the constant regions of the H and L chain genes. A simpler method has been developed that uses the PCR. From a comparison of a large number of antibody sequences, amino acids frequently found at the amino termini of antibodies were identified. From this information, a set of degenerate PCR primers was designed that correspond to all the possible sequences in this region. Because the amino acids in the constant domains of different antibodies are nearly identical, only one PCR primer is needed for the 3′ end of each H and L chain sequence. To directly clone the antibody cDNAs, cDNA is prepared by treating hybridoma mRNA with reverse transcriptase, mixed with a pair of PCR primers (in this case, for amplifying the heavy chain sequences), and subjected to PCR. Without knowledge of the amino terminus of the antibody chain, a PCR had to be set up with each of the different 5′ primers until an amplified DNA fragment was obtained. The process can be simplified if the sequence of the first six or seven amino acids of the antibody can be determined; this is sufficient to design a single 5′ PCR primer.

Enzymes catalyze reactions by stabilizing a chemical structure intermediate between the substrate and product, termed the *transition state*. Thus, if monoclonal antibodies could be made to a transition state analogue—a molecule resembling the transition state of a chemical reaction—then some of these antibodies might have catalytic activity. The ability to produce custom-designed catalysts would be very valuable, especially to the chemical and pharmaceutical industries.

Initial attempts to produce catalytic antibodies indicated that they were exceedingly rare and often not found among the hybridomas produced by conventional monoclonal antibody technology. An excellent fusion might produce several hundred different antibodies, but the entire repertoire of antibodies that can be produced by the immune system is perhaps 100 million. How can the entire repertoire be tapped?

One strategy that shows promise is to bypass the inefficient fusion step in hybridoma production and directly clone antibody cDNAs from the lymphocytes of immunized mice (Figures 23-6 and 23-7). Investigators inoculated a mouse with an antigen. They recovered spleen cells from the mouse and used PCR to amplify millions of cDNAs for antibody light and heavy chains. The light- and heavy-chain cDNAs were cloned separately into phage vectors and then recombined in vitro to generate a third, *combinatorial* library of phage carrying random pairs of light and heavy chains. The library was plated onto a bacterial lawn, and the resulting phage plaques, each containing a unique antibody, were screened with radioactively labeled antigen in a manner similar to that used for

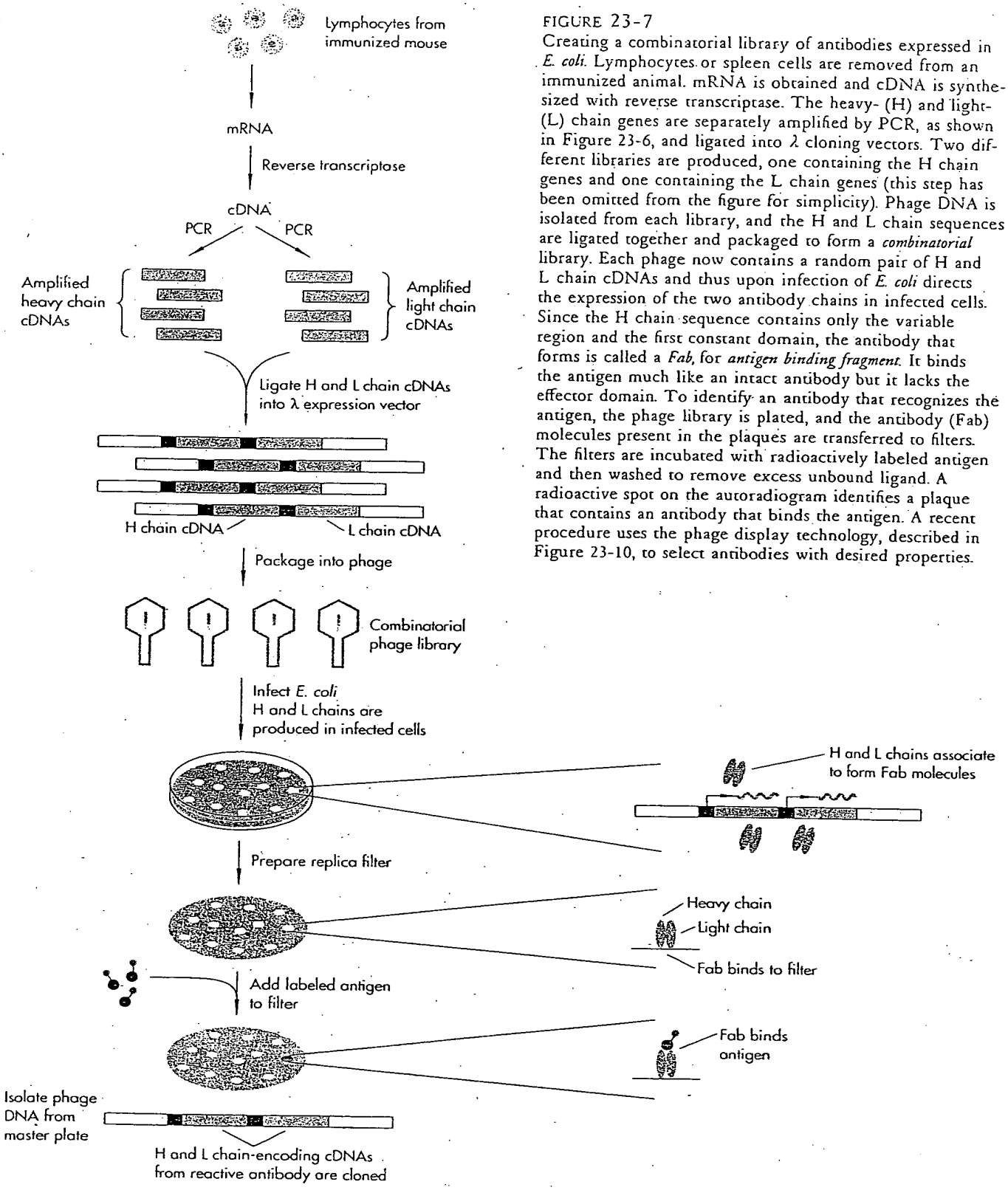


FIGURE 23-7
Creating a combinatorial library of antibodies expressed in *E. coli*. Lymphocytes or spleen cells are removed from an immunized animal. mRNA is obtained and cDNA is synthesized with reverse transcriptase. The heavy- (H) and light- (L) chain genes are separately amplified by PCR, as shown in Figure 23-6, and ligated into λ cloning vectors. Two different libraries are produced, one containing the H chain genes and one containing the L chain genes (this step has been omitted from the figure for simplicity). Phage DNA is isolated from each library, and the H and L chain sequences are ligated together and packaged to form a *combinatorial* library. Each phage now contains a random pair of H and L chain cDNAs and thus upon infection of *E. coli* directs the expression of the two antibody chains in infected cells. Since the H chain sequence contains only the variable region and the first constant domain, the antibody that forms is called a *Fab*, for *antigen binding fragment*. It binds the antigen much like an intact antibody but it lacks the effector domain. To identify an antibody that recognizes the antigen, the phage library is plated, and the antibody (Fab) molecules present in the plaques are transferred to filters. The filters are incubated with radioactively labeled antigen and then washed to remove excess unbound ligand. A radioactive spot on the autoradiogram identifies a plaque that contains an antibody that binds the antigen. A recent procedure uses the phage display technology, described in Figure 23-10, to select antibodies with desired properties.

cloning cDNAs from an expression library (Figure 7-10). Out of a million phage plaques screened, 200 clones were identified that produced an antibody binding the antigen. Thus, with this approach, investigators were able to sample a million possible antibodies—at least a thousand times more than they could screen by conventional monoclonal antibody technology. Since phages in a particular plaque encode the antibody expressed in the plaque, it is a trivial matter to clone the heavy- and light-chain cDNAs from the phage DNA. These cDNAs can be placed into bacterial or mammalian expression vectors for production of large quantities of the selected antibody.

A recent modification of this method uses filamentous phages such as M13 instead of λ phage and allows display of the antibodies on the phage surface. This offers the advantage of being able to screen thousands more phage (because the screening can be done in solution) and to select phage that express tight-binding antibodies. We will discuss this method later and in Figure 23-10.

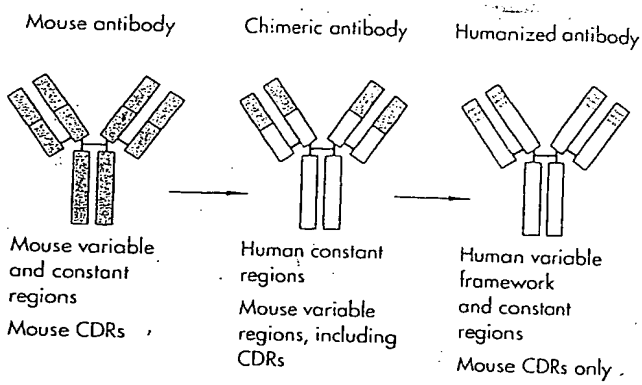


FIGURE 23-8
Antibody engineering. The basic structure of a mouse monoclonal antibody (MAb) resembles that of a human antibody. However, there are numerous differences between amino acid sequences of the antibodies from the two species. These sequence differences account for the immunogenicity of mouse MAbs in humans. A *chimeric* MAb is constructed by ligating the cDNA fragment encoding the mouse V_L and V_H domains to fragments encoding the C domains from a human antibody. Because the C domains do not contribute to antigen binding, the chimeric antibody will retain the same antigen specificity as the original mouse MAb but will be closer to human antibodies in sequence. Chimeric MAbs still contains some mouse sequences, however, and may still be immunogenic. A *humanized* MAb contains only those mouse amino acids necessary to recognize the antigen. This product is constructed by building into a human antibody the amino acids from the mouse complementarity determining regions or CDRs.

"Humanized" Monoclonal Antibodies Retain Activity But Lose Immunogenicity

Although swift progress is being made in the identification of monoclonal antibodies with potential therapeutic value, their use is limited by a problem we have already discussed in this chapter. Monoclonal antibodies are usually mouse proteins, and they are not identical to human antibodies. Thus, antibodies injected into a patient will eventually be recognized as foreign proteins and will be cleared from the circulation.

As we learned in Chapter 16, both chains of the antibody molecule can be divided into variable and constant regions. The variable regions differ in sequence from one antibody to another, and this is the region of the protein that binds the antigen. The constant region is the same among all antibodies of the same type. The first method used to reduce the immunogenicity of a mouse monoclonal antibody was simply to construct *chimeric* genes that encoded proteins in which the variable regions from the mouse antibody were fused to the constant regions from a human antibody. The chimeric antibody (Figure 23-8) retained its binding specificity but more closely resembled a natural human antibody.

This antibody, however, was not fully *humanized*, because it retained amino acid sequences from the mouse protein. Thus, scientists have set out to engineer fully humanized monoclonal antibodies that will be indistinguishable from natural molecules. Extensive studies of the three-dimensional structures of antibody molecules tell us that only a few of the one hundred amino acids in the variable region of an antibody actually contact the antigen; these regions of contact are referred to as *complementarity determining regions* (CDRs). Three CDRs each comprise the antigen-binding sites on the light and heavy chains. The rest

of the variable region serves as a scaffold to anchor the CDRs in the correct positions. This breakdown of amino acids in the variable region into those serving recognition and those serving structural roles is also evident from simply comparing the sequences of many antibody molecules. Amino acid sequences in the CDRs are *hypervariable*, whereas the structural, or *framework*, amino acids differ little.

Thus, to make a fully humanized antibody, all that would be required in principle would be to use in vitro mutagenesis to transfer the CDR amino acid sequences from a mouse MAb to a natural human antibody (Figure 23-8). This method was used to humanize an antibody that recognizes an antigen on the surface of human lymphocytes. This humanized MAb is now in clinical trials as an immunosuppressant and for treatment of lymphoid tumors. Another potentially valuable MAb binds a growth factor receptor found in large numbers on the surface of many breast tumor cells. Laboratory experiments showed that this antibody could block the growth of these cells in culture and caused tumors seeded in mice to regress. Unfortunately, the first humanized versions of this antibody bound the receptor protein but failed to block the growth of breast carcinoma cells. Investigators suspected that the problem was with the framework amino acids, and they used computer modeling to design amino acid substitutions that would strengthen the antibody-antigen interaction. Several such variant antibodies were produced and tested; one bound the receptor 250 times more tightly than did the original antibody and successfully blocked tumor cell growth in culture. This antibody is now being produced in large quantities for clinical trials.

Protein Engineering Can Tailor Antibodies for Specific Applications

Humanizing monoclonal antibodies is an example of the emerging technology of *protein engineering*, that is, a process using recombinant DNA to modify the structure of natural proteins to improve or change their function. Antibodies are particularly attractive candidates for protein engineering, because their structure is understood in great detail and because their potential for use in medicine is enormous. Another way in which antibodies are being engineered is by changing their *effector domains*, the regions of the heavy chain that specify antibody function—for example, killing of cells marked by the antibody. In this way, the mode of action of a monoclonal antibody can be reprogrammed. One promising strategy is to replace the effector domain entirely with a sequence encoding a toxin. An antibody-toxin fusion protein would deliver the toxin specifically to cells bearing the target antigen. This product could be an exceptionally potent treatment for cancer and for viral diseases such as AIDS. Antibody engineering is also being used to construct *bispecific antibodies*. In these antibodies, each of the two arms recognizes a different antigen, thus allowing an antibody to bridge the two antigens. For example, a bispecific antibody could recognize a tumor cell protein with one arm and a protein on the surface of a killer T cell with the other, thereby bringing the killer cells directly to the tumor (Figure 23-9).

Protein Engineering Is Used to Improve a Detergent Enzyme

Subtilisin is a serine protease produced by bacteria. Due to its broad specificity for proteins that commonly soil clothing, this enzyme was developed for commercial use in laundry detergents. (It is subtilisin that is prominently advertised as the enzyme additive in modern detergents.) But the first detergents containing subtilisin suffered from a serious drawback: they could not be used with bleach, because bleach inactivates the enzyme. Biochemical analysis determined that loss of activity was due to the oxidation of a methionine at position 222. Once this happened, the modified enzyme lost 90 percent of its activity. Because they knew which amino acid was bleach sensitive, however, scientists decided to see whether a variant of subtilisin could be produced that was no longer sensitive to bleach.

To do this, site-directed mutants were constructed in the gene encoding subtilisin. The strategy was simply to substitute, one at a time, each of the non–wild-type amino acids at residue 222. The mutant genes

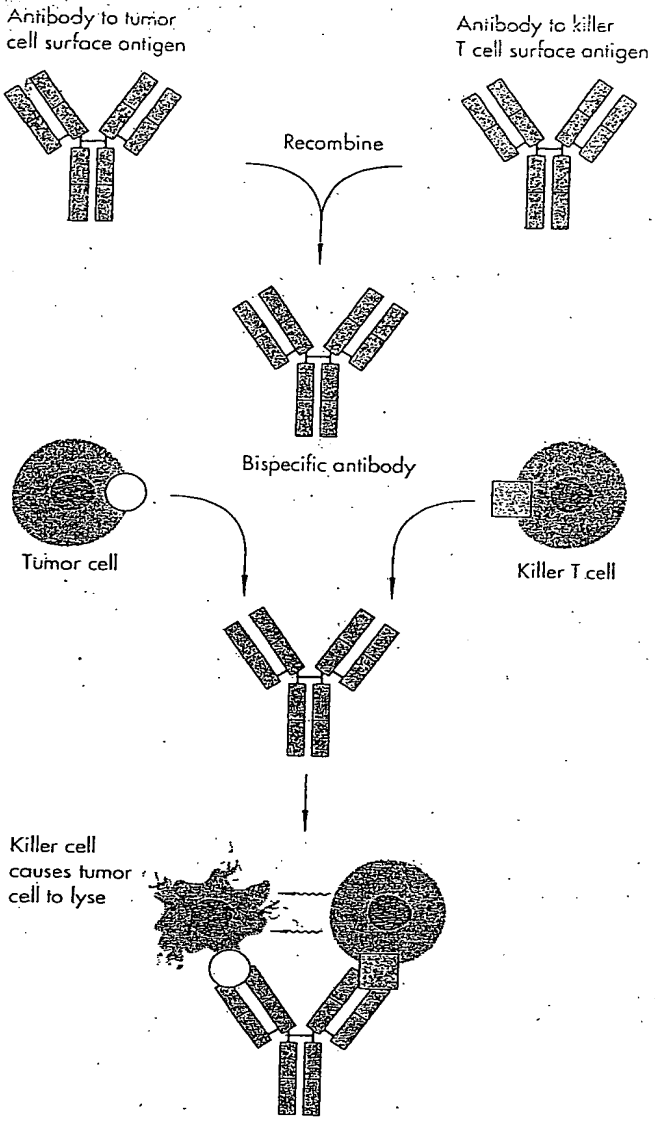


FIGURE 23-9
A bispecific antibody. By using recombinant DNA, the cDNAs for antibodies to two different antigens can be engineered to make an antibody in which each arm recognizes a different antigen. Thus it is possible to recombine antibodies to surface antigen on tumor cells and to a protein on cytotoxic T cells to make a bispecific antibody that brings the two cells together to facilitate killing of the tumor cells.

were cloned into expression vectors and the 19 different subtilisin derivatives were expressed. Biochemical analysis showed that the cysteine-222 enzyme was even more active than the wild-type protein, but it was also inactivated by bleach. The next most active variant was the alanine-substituted enzyme, which was 53 percent as active as wild-type subtilisin. This variant exhibited no detectable bleach sensitivity, so detergents containing this engineered subtilisin can now be used with bleach. This new variant of subtilisin is an example of a *second-generation* molecule, a molecule specifically engineered for a new desirable trait. Protein engineers are currently at work on a third-generation molecule that exhibits decreased temperature sensitivity so that it can be used in hot water.

This experiment points out the power of recombinant DNA as a tool for the engineering of natural products. Changing the properties of a protein was all but impossible prior to the development of recombinant DNA techniques. Now it is not only possible, but easy. It is a routine exercise for protein engineers to generate hundreds of variants of a natural protein for testing. These changes can be educated guesses based on detailed knowledge of the structure of a protein; alternatively, changes can easily be made on a purely random basis. And, as we will see in the next section, a combination of structural information with random mutagenesis and a powerful selection for improved protein function can have dramatic results.

Growth Hormone Variants with Improved Binding Are Selected by Phage Display

To engineer an improved subtilisin enzyme, researchers were aided by the knowledge that only one specific amino acid had to be changed. Thus, they could systematically vary that amino acid to find the one that worked the best. But more complex challenges face protein engineers. Is it possible, for example, to engineer antibodies with higher affinity for antigen; to design an inhibitor that tightly binds to and blocks a cell-surface protein or an enzyme inside a cell; to generate a growth factor or hormone with increased affinity for its receptor? Alterations of this sort require several amino acid changes, and with 20 possible amino acids at each position, the number of variants that

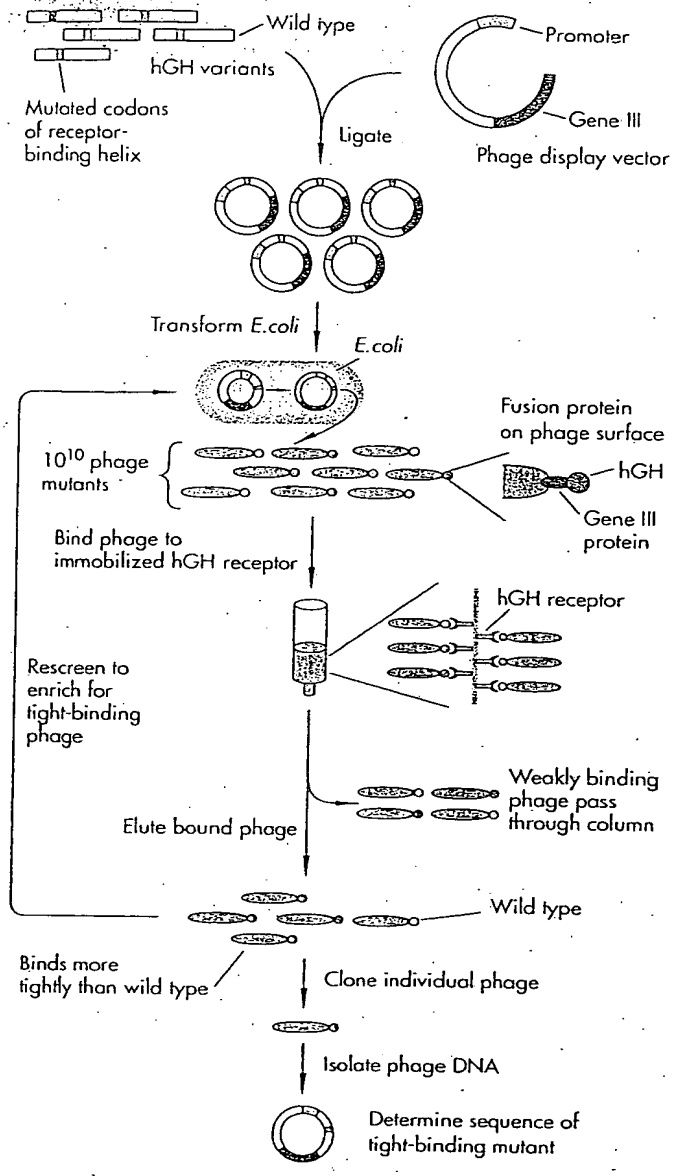


FIGURE 23-10
Expression of proteins and peptides on the surface of filamentous phage. A library of randomly mutated hGH cDNAs was ligated into an M13-based phagemid vector so that hGH was fused to the carboxy-terminal domain of the M13 gene III protein. The carboxy terminus of the gene III protein associates with the phage particle, and the amino terminus, containing the hGH variants, is displayed on the outer surface of the phage. The library of phagemids is introduced into *E. coli*, and ampicillin-resistant colonies are obtained. These *E. coli* are then infected with a helper phage that induces the production of phagemid particles. Only 1–10 percent of the phage particles contain an hGH–gene III fusion protein, and these contain only one hGH fusion molecule per phage. This ensures that the phage retain sufficient wild-type gene III protein in their coats to remain infectious. hGH-phage were passed through a column containing the hGH receptor covalently linked to plastic beads. Only the phage expressing hGH were retained. The nonbinding phage lacking hGH passed through the column. The bound phage were isolated, cultured in *E. coli*, and passed again over the column. Repeated rounds of selection resulted in the identification of hGH variants that bound the receptor with exceptionally high affinity.

need to be screened is enormous (for changes at just 3 amino acids, there are 8000 different combinations; for 10 amino acids, 10^{13} different proteins are possible). Clearly, these variants cannot be made and tested one at a time, and a method for direct selection of improved proteins is needed.

Researchers have used a new approach to select variants of human growth hormone with increased affinity for growth hormone receptor (Figure 23-10). From structural studies and extensive mutagenesis of hGH, they knew what portions of the amino acid sequence were important for receptor binding. They synthesized degenerate oligonucleotides that encoded all possible amino acids at these positions and ligated the pool of oligonucleotides in place of the natural hGH sequence. The resulting pool of variant hGH cDNAs was fused to the reading frame of gene III in the filamentous phage M13. Gene III encodes a minor phage coat protein expressed on the surface of the phage, and incorporation of the hGH cDNA into this gene results in the display of the hGH variants on the surface of the phage, one variant per phage. This technique is known as *phage display*.

Now it was a simple matter to pass this library of more than 10^{11} different phage over a column containing the hGH receptor. Phage displaying weakly binding hGH variants were washed off the column, and phage displaying tightly binding variants were recovered with a more stringent wash. This population of tight-binding phage was amplified by infection of *E. coli* and passed over the column a second time. The selection was repeated for a total of six rounds, each round enriching for the phage displaying hGH variants

with highest affinity for the receptor bound to the column. At this point, individual phage were cloned, the affinities of their hGH variants were measured directly, and the sequences of the hGH cDNAs were examined. Among these variants was one that bound its receptor about 10 times more tightly than natural hGH did. When selected amino acids from another region of hGH that had been randomized were introduced into this variant, the resulting hGH molecule bound to the hGH receptor over 50 times more tightly than the wild-type hGH did. This process is being repeated in the hope to obtain even more tightly binding variants.

The ability afforded by techniques such as phage display to correlate protein structure and function in a systematic way makes possible new methods of finding novel drugs. If researchers have a good idea what combination of amino acids gives the best fit to the binding site on a receptor, the next step in *rational drug design* would be to design, or even select, a small peptide that binds as well as the larger protein. And then, using computer modeling to display the molecular contacts between ligand and receptor, researchers can attempt to design and synthesize small nonprotein molecules that make the same contacts. The end-product would be a small organic molecule that could be produced more cheaply than a recombinant protein, yet would retain the full biological activity of the protein hormone. And, more important, such molecules could be administered orally, thus eliminating the major disadvantage of most recombinant protein therapeutics—that they must be delivered directly into the bloodstream by injection. This type of rational drug design contrasts sharply with the conventional approach to drug discovery now in use in the pharmaceutical industry, in which an inventory of completely unrelated compounds is tested at random until an active compound is found.

New Technologies Promise New Approaches to Drug Design

The biotechnology industry is in its infancy, and its successes to date follow directly from developments in molecular biology that are already nearly two decades old. The recombinant drugs currently in clinical use arise from what is by now conventional technology—gene cloning, expression, and mutagenesis to improve protein function. These methods will continue to turn out new drugs such as erythropoietins to treat anemia caused by kidney disease, DNase to treat cystic fibrosis, or colony-stimulating factors (CSFs) to increase white blood cell production during chemotherapy.

But the true promise of biotechnology is in novel technologies that are only now being developed. We have mentioned efforts to design catalytic antibodies that can accelerate chemical reactions in both medical and industrial applications. This is but one example of a whole new approach to protein engineering in which novel activities can be placed on unrelated protein scaffolds, using random mutagenesis coupled with selection methods like phage display. Similar goals may be achieved by the engineering of *ribozymes*, RNA molecules with catalytic activity, and the use of the polymerase chain reaction to select nucleic acid molecules that bind tightly to targets of medical importance. Another strategy that may see widespread application is treatment with antisense DNA and RNA to inhibit the expression of oncogenes in tumors or of viral genes in infected patients. And a variety of new technologies based on viral vectors promise new approaches for vaccines and gene therapy.

Many of these techniques now work in the test tube, and the principal challenge facing biotechnology companies is to turn these laboratory techniques into commercially viable processes.

Reading List

General

Hall, S. S. *Invisible Frontiers: The Race to Synthesize a Human Gene.* Atlantic Monthly Press, New York, 1987.

Hood, L. "Biotechnology and medicine of the future." *J. Am. Med. Assoc.*, 259: 1837–1844 (1988).

Goeddel, D. V. (ed.) Systems for Heterologous Gene Expression. *Meth. Enzymol.*, Vol. 185, Academic Press, New York, 1990.

Original Research Papers

EXPRESSION OF HUMAN PROTEINS IN E. COLI

Itakura, K., T. Hirose, R. Crea, A. Riggs, H. L. Heyneker, F. Bolivar, and H. Boyer. "Expression in *E. coli* of a chemically synthesized gene for the hormone somatostatin." *Science*, 198: 1056–1063 (1977).

Goeddel, D. V., H. L. Heyneker, T. Hozumi, R. Arentzen, K. Itakura, D. G. Yansura, M. J. Ross, G. Miozzari, R. Crea, and P. Seeburg. "Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone." *Nature*, 281: 544–548 (1979).

Goeddel, D. V., D. G. Kleid, F. Bolivar, H. L. Heyneker, D. G. Yansura, R. Crea, T. Hirose, A. Kraszewski, K. Itakura, and A. D. Riggs. "Expression of chemically synthesized genes for human insulin." *Proc. Natl. Acad. Sci. USA*, 76: 106–110 (1979).

EXPRESSION IN YEAST

Valenzuela, P., A. Medina, W. J. Rutter, G. Ammerer, and B. D. Hall. "Synthesis and assembly of hepatitis B virus surface antigen particles in yeast." *Nature*, 298: 347–350 (1982).

Hitzeman, R. A., D. W. Leung, L. J. Perry, W. J. Kohr, H. L. Levine, and D. V. Goeddel. "Secretion of human interferons by yeast." *Science*, 219: 620–625 (1983).

Sabin, E. A., C. T. Lee-Ng, J. R. Shuster, and P. J. Barr. "High-level expression and in vivo processing of chimeric ubiquitin fusion proteins in *Saccharomyces cerevisiae*." *Bio/Tech.*, 7: 705–709 (1989).

EXPRESSION IN INSECT CELLS

Luckow, V. A., and M. D. Summers. "Trends in the development of baculovirus expression vectors." *Bio/Tech.*, 6: 47–55 (1988). [Review]

Medin, J. A., L. Hunt, K. Gathy, R. K. Evans, and M. S. Coleman. "Efficient, low-cost protein factories: expression of human adenosine deaminase in baculovirus-infected insect larvae." *Proc. Natl. Acad. Sci. USA*, 87: 2760–2764 (1990).

EXPRESSION OF PROTEINS IN MAMMALIAN CELLS

Gorman, C. M. "Mammalian cell expression." *Curr. Opin. Biotech.*, 1: 36–43 (1990). [Review]

Pennica, D., W. E. Holmes, W. J. Kohr, R. N. Harkins, G. A. Vehar, C. A. Ward, W. F. Bennett, E. Yelverton, P. H. Seeburg, H. L. Heyneker, D. V. Goeddel, and D. Collen. "Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*." *Nature*, 301: 214–221 (1983).

Paborsky, L. R., B. M. Fendly, K. L. Fisher, R. M. Lawn, B. J. Marks, G. McCray, K. M. Tate, G. A. Vehar, and C. M. Gorman. "Mammalian cell transient expression of tissue factor for the production of antigen." *Prot. Eng.*, 3: 547–553 (1990).

VACCINES

Brown, F. "From Jenner to genes—the new vaccines." *Lancet*, 335: 587–590 (1990). [Review]

Bolognesi, D. P. "Approaches to HIV vaccine design." *Trends Biotech.* 8: 40–45 (1990). [Review]

Berman, P. W., T. J. Gregory, L. Riddle, G. R. Nakamura, M. A. Champe, J. P. Porter, F. M. Wurm, R. D. Hershberg, E. K. Cobb, and J. W. Eichberg. "Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160." *Nature*, 345: 622–625 (1990).

PROTEIN ENGINEERING RECOMBINANT PRODUCTS

Estell, D. A., T. P. Graycar, and J. A. Wells, "Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation." *J. Biol. Chem.* 260: 6518–6521 (1985).

DesJarlais, R. L., G. L. Seibel, I. D. Kuntz, P. S. Furth, J. C. Alvarez, P. R. Ortiz de Montellano, D. L. DeCamp, L. M. Babe, and C. S. Craik. "Structure-based design of nonpeptide inhibitors specific for the human immunodeficiency virus 1 protease." *Proc. Natl. Acad. Sci. USA*, 87: 6644–6648 (1990).

Abrahmsen, L., J. Tom, J. Burnier, K. A. Butcher, A. Kossiakoff, and J. A. Wells. "Engineering subtilisin and its substrates for efficient ligation of peptide bonds in aqueous solution." *Biochemistry*, 30: 4151–4159 (1991).

Bennett, W. F., N. F. Paoni, B. A. Keyt, D. Botstein, A. J. S. Jones, L. Presta, F. M. Wurm, and M. J. Zoller. "High resolution analysis of functional determinants on human tissue-type plasminogen activator." *J. Biol. Chem.*, 266: 5191–5201 (1991).

Cunningham, B. C. and J. A. Wells. "Rational design of receptor-specific variants of human growth hormone." *Proc. Natl. Acad. Sci. USA*, 88: 3407–3411 (1991).

MONOCLONAL ANTIBODIES

Milstein, C. "Monoclonal antibodies." *Sci. Am.*, 243: 66–74 (1980). [Review]

CLONING AND EXPRESSION OF ANTIBODIES

Pluckthun, A. "Antibodies from *Escherichia coli*." *Nature*, 347: 497–498 (1990). [Review]

Huse, W. D., L. Sastry, S. A. Iverson, A. S. Kang, M. Alting-Mies, D. R. Burton, S. J. Benkovic, and R. A. Lerner. "Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda." *Science*, 246: 1275–1289 (1989).

Chaudhary, V. K., J. K. Batra, M. G. Gallo, M. C. Willingham, D. J. FitzGerald, and I. Pastan. "A rapid method of cloning functional variable-region antibody genes in *Escherichia coli* as single-chain immunotoxins." *Proc. Natl. Acad. Sci. USA*, 87: 1066–1070 (1990).

Mullinax, R. L. et al. "Identification of human antibody fragment clones specific for tetanus toxoid in a bacteriophage λ immunoexpression library." *Proc. Natl. Acad. Sci. USA*, 87: 8095–8099 (1990).

Berg, J., E. Lotscher, K. S. Steimer, D. J. Capon, J. Baenziger, H. M. Jack, and M. Wabl. "Bispecific antibodies that mediate killing of cells infected with human immunodeficiency virus of any strain." *Proc. Natl. Acad. Sci. USA*, 88: 4723–4727 (1991).

Wood, C.R., A. J. Dorner, G. E. Morris, E. M. Alderman, D. Wilson, R. M. J. O'Hara, and R. J. Kaufman. "High level synthesis of immunoglobulins in Chinese hamster ovary cells." *J. Immunol.*, 145: 3011–3016 (1990).

CATALYTIC ANTIBODIES

Jacobs, J. W. "New perspective on catalytic antibodies." *Bio/Tech.*, 9: 258–262 (1991). [Review]

Benkovic, S. J., J. A. Adams, C. L. Borders, K. D. Janda, and R. A. Lerner. "The enzymic nature of antibody catalysis: development of multistep kinetic processing." *Science*, 250: 1135–1139 (1990).

Bowdish, K., Y. Tang, J. B. Hicks, and D. Hilvert. "Yeast expression of a catalytic antibody with chorismate mutase activity." *J. Biol. Chem.*, 266: 11901–11908 (1991).

HUMANIZING ANTIBODIES

Winter, G., and C. Milstein. "Man-made antibodies." *Nature*, 349: 293–299 (1991). [Review]

Jones, P. T., P. H. Dear, J. Foote, M. S. Neuberger, and G. Winter. "Replacing the complementarity-determining regions in a human antibody with those from a mouse." *Nature*, 321: 522–525 (1986).

Riechmann, L., M. Clark, H. Waldmann, and G. Winter. "Reshaping human antibodies for therapy." *Nature*, 332: 323–327 (1988).

Carter, P., L. Presta, C. M. Gorman, J. B. Ridgway, D. Henner, W. L. T. Wong, A. M. Rowland, C. Kotts, M. E. Carver, and M. H. Sheppard. "Humanization of an anti-p185^{HER2} antibody for human cancer therapy." *Proc. Natl. Acad. Sci. USA*, 89: In press (1992).

PHAGE DISPLAY

Smith, G. P. "Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface." *Science*, 228: 1315–1317 (1985).

Cwirla, S. E., E. A. Peters, R. W. Barrett, and W. J. Dower. "Peptides on phage: a vast library of peptides for identifying ligands." *Proc. Natl. Acad. Sci. USA*, 87: 6378–6382 (1990).

Clackson, T., H. R. Hoogenboom, A. D. Griffiths, and G. Winter. "Making antibody fragments using phage display libraries." *Nature*, 352: 624–628 (1991).

Lowman, H. B., S. Bass, N. Simpson, and J. A. Wells. "Selecting high affinity binding proteins by monovalent phage display." *Biochemistry*, 30, 10832–10838 (1991).

NEW RECOMBINANT DRUGS

Shak, S., D. J. Capon, R. Hellmiss, S. A. Marsters, and C. L. Baker. "Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum." *Proc. Natl. Acad. Sci. USA*, 87: 9188–9192 (1990).

Takaue, Y., et al. "Effects of recombinant human G-CSF, GM-CSF, IL-3, and IL-1 alpha on the growth of purified human peripheral blood progenitors." *Blood*, 76: 330–335 (1990).

Shepard, H.M., G. D. Lewis, J. C. Sarup, B. M. Fendly, D. Maneval, J. Mordenti, I. Figari, C. E. Kotts, M. A. Palladino, A. Ullrich, and D. slamon. "Monoclonal antibody therapy of human cancer: taking the HER2 protooncogene to the clinic." *J. Clin. Immunol.*, 11: 117–127 (1991).

Watson, S. R., C. Fennie, and L. A. Lasky. "Neutrophil influx into an inflammatory site inhibited by a soluble homing receptor-IgG chimaera." *Nature*, 349: 164–167 (1991).

FUTURE TRENDS

Sarver, N., E. M. Cantin, P. S. Chang, J. A. Zaia, P. A. Ladne, D. A. Stephens, and J. J. Rossi. "Ribozymes as potential anti-HIV-1 therapeutic agents." *Science*, 247: 1222–1225 (1990).

Tuerk, C., and L. Gold. "Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase." *Science*, 249: 505–510 (1990).

Han, L., J. S. Yun, and T. E. Wagner. "Inhibition of Moloney murine leukemia virus-induced leukemia in transgenic mice expressing antisense RNA complementary to the retroviral packaging sequences." *Proc. Natl. Acad. Sci. USA*, 88: 4313–4317 (1991).

Miller, P. S. "Oligonucleoside methylphosphonates as antisense reagents." *Bio/Tech.*, 9: 358–362 (1991).

Schultz, J. S. "Biosensors." *Sci. Am.*, 265: 64–69 (1991).